Vol. XXXIX, No. 6 (December, 1958), was issued on 16.12.58.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XL FEBRUARY, 1959 NO. 1

EFFECT OF ADJUVANT (FREUND'S TYPE) AND ITS COMPONENTS ON THE ORGANS OF VARIOUS ANIMAL SPECIES. A COMPARATIVE STUDY*

A. LAUFER, CHLOE TAL AND A. J. BEHAR

From the Department of Pathology, and the Department of Nervous Diseases, Laboratory of Experimental Neurology, The Rothschild-Hadassah University Hospital and the Hebrew University-Hadassah Medical School, Jerusalem, Israel

Received for publication July 16, 1958

In a previous publication dealing with the production of allergic encephalitis in the Syrian hamster, it was noted that in the control group of animals injected with Freund's adjuvant alone a granulomatous proliferation of the reticulo-endothelial cells occurred in the brain (Tal, Laufer and Behar, 1958). This finding induced us to investigate the reaction of the brain to the same agent and its components in other animal species. Likewise, the reaction of organs other than the brain to the injection of complete adjuvant or of its components was studied.

MATERIALS AND METHODS

Three species of animals were used : guinea-pigs, mice and Syrian hamsters.

Mice were W-Swiss H line, inbred, 6 weeks old, both sexes, weighing 20–25 g. It is known that in animals of this line allergic encephalitis can be readily produced. Guinea-pigs of mixed breed, from the stock of the Hebrew University, Jerusalem, 2–3 months old, both sexes, weighing 350–450 g. Syrian hamsters from the Hebrew University stock, 3 months old, both sexes, weighing 80 g. each.

The Freund's adjuvant injected was the same as that used in the previous study (Freund and McDermett, 1942 ; Olitsky and Tal, 1952).

The animals of each species were divided into 3 groups as follows :

Group I consisted of animals which received the complete adjuvant.

Group II consisted of animals which received only the oil fraction of the adjuvant. This fraction was prepared by mixing 50 ml. of Byol F., 50 ml. of physiological saline and 20 mg. of merthiolate in a Waring blender for 5 min.

Group III consisted of animals injected with the tuberculous fraction of the adjuvant, prepared by mixing 250 mg. of autoclaved human *Mycobacterium tuberculosis* from the Lederle Laboratory, New York, 20 mg. of merthiolate and 100 ml. of physiological saline in a Waring blender for 5 min.

* This study was supported partly by the Hebrew University-Hadassah Medical School Research Fund, and partly by the Julius and Marie Scheider Neuropsychiatric Fund.

The 3 animal species were injected with the same batches of material, all of which were tested for sterility.

The number of animals used is shown in Table IV. Each mouse received a subcutaneous injection of 0·3 ml. of the respective substance once weekly for 6 weeks. The same applied to the hamsters except that each injection amounted to 1·0 ml. The animals were observed daily and were sacrificed 5 months after the first injection.

The guinea-pigs each received a single injection of 3·0 ml. of the respective substance into the muscles of the back of the neck. They, too, were observed daily and were sacrificed after 5 months.

All animals were killed by ether inhalation.

For histological examination blocks of tissue were taken from the following organs: brain, liver, spleen, lungs, heart and kidneys. These were fixed in 10 per cent neutral formalin and were then embedded in paraffin. Brain sections were stained with haematoxylin and eosin, by the Weil-Weigert and Luxol fast-blue methods for myelin. Sections of the other organs were stained with haematoxylin and eosin, Gentian violet, Congo red and P.A.S.

RESULTS

Mice

Group I.—Injected with complete adjuvant

Histological changes were found in the liver, spleen, kidneys and lungs. The brain, as well as the heart, in these animals showed no changes.

Liver.—In 17 animals the liver showed proliferative activity of reticulo-endothelial cells, and in 5 of these amyloidosis was also found. The intensity of the proliferative process varied. The lesions consisted of round mononuclear and histiocytic cells in the portal spaces or around central veins and in some areas extended between the liver plates (Fig. 1 and 1A). Here and there small foci of a similar structure were seen extending into the parenchyma. Rarely, a few polymorphonuclear cells were present in the cellular proliferations and sometimes a few giant cells were seen. Amyloid, when present, stained with Gentian violet and Congo red, and was deposited along the sinusoidal walls. Atrophy of the liver parenchyma was present and in some locations the parenchyma was completely

---

EXPLANATION OF PLATES

FIG. 1.—Proliferation of reticulo-endothelial elements in a portal space and around a central vein. × 111. H. and E.

FIG. 1A.—Reticulo-endothelial proliferation in the liver: Histiocytes, lymphoid cells, scanty leucocytes. × 404. H. and E.

FIG. 2.—Amyloid deposits along the sinusoidal wall with atrophy of liver trabeculae. Note the reticulo-endothelial proliferation around the central vein. × 111. H. and E.

FIG. 3.—Large deposits of amyloid in spleen. × 111. H. and E.

FIG. 4.—Amyloidosis in glomeruli of kidneys. × 111. H. and E.

FIG. 5.—Proliferation of reticulo-endothelial elements around the central vein extending between the liver trabeculae. × 148. H. and E.

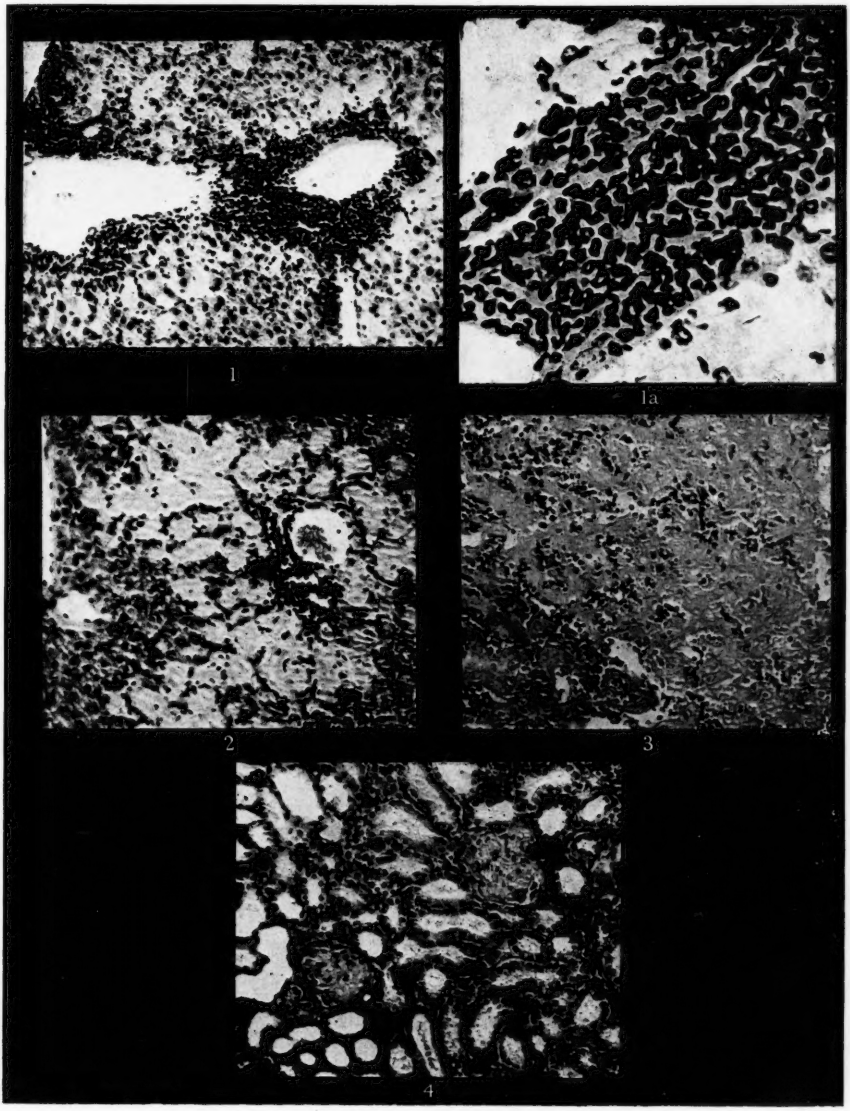
FIG. 6.—Granulomatous lesion in the lung. × 297. H. and E.

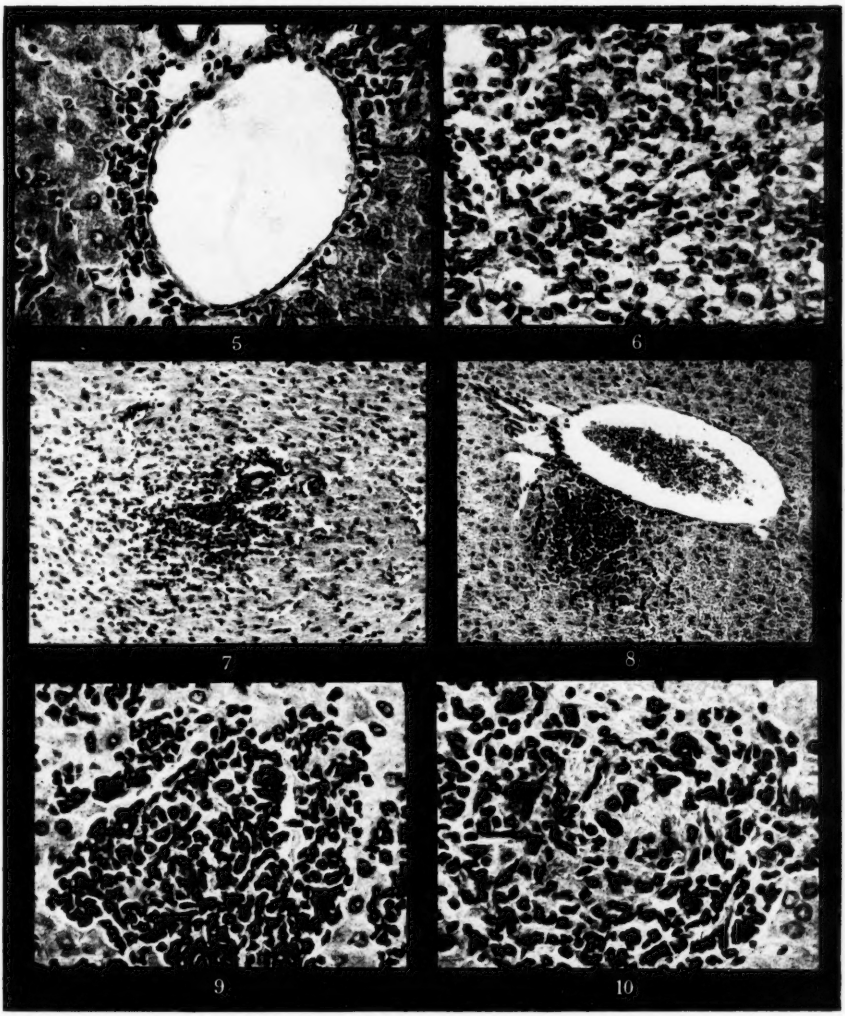
FIG. 7.—Perivascular and juxtavascular inflammatory proliferative focus in brain. × 148. H. and E.

FIG. 8.—Reticulo-endothelial proliferation around a central vein extending into the liver parenchyma. × 99. H. and E.

FIG. 9.—The cellular elements of a focus in the liver; histiocytes, lymphocytes and rare leucocytes. × 370. H. and E.

FIG. 10.—Granuloma with epithelioid cells and giant cells in liver. × 370. H. and E.


1
1a
2
3
4



Laufer, Chloe Tal and Behar.

replaced by amyloid (Fig. 2). A similar deposit of amyloid was found in the walls of some vessels. In many instances cellular proliferations were in close proximity to amyloid deposits.

Spleen.—The general architectural pattern of the spleen was usually preserved. The histological picture varied. In many sections a marked proliferation of the cells of the red pulp was present with plasma cells, histiocytes and numerous giant cells in evidence. These changes were found in the spleen in 11 animals ; in addition

TABLE I.—*Summary of Histological Findings in Mice*

Material injected	No. of animal	Brain	Liver	Spleen	Kidney	Heart	Lung
Complete adjuvant	1	—	+	+	—	—	Pneumonia.
	2	—	+	+	—	—	—
	3	—	+	+	—	—	Pneumonia.
	4	—	+	+	—	—	—
	5	—	+	+	—	—	Granuloma around bronchi.
	6	—	+	+	—	—	Granulomata.
	7	—	—	AM	—	—	—
	8	—	—	+	—	—	—
	9	—	+	—	—	—	—
	10	—	+	—	—	—	—
	11	—	+	—	—	—	Granuloma.
	12	—	+	+AM	—	—	Proliferation of adventitial cells.
	13	—	+	—	—	—	Granulomata.
	14	—	—	+	—	—	Granuloma.
	15	—	+AM	AM	—	—	—
	16	—	+AM	AM	AM	—	—
	17	—	+AM	AM	AM	—	—
	18	—	+AM	AM	AM	—	Foci of perivascular cuffing.
	19	—	+AM	—	AM	—	—
	20	—	—	—	—	—	Granuloma.
	21	—	+	—	—	—	Bronchopneumonia.
	22	—	—	+AM	—	—	—
	23	—	—	+	—	—	—
	24	—	—	—	—	—	—
	25	—	—	—	—	—	—
Oil fraction of adjuvant	1	—	—	+AM	—	—	—
	2	—	—	—	—	—	—
	3	—	+AM	+AM	—	—	—
	4	—	+	+	—	—	—
	5	—	—	+AM	—	—	—
	6	—	+	—	—	—	—
	7	—	+	—	—	—	—
	8	—	+	+	—	—	—
	9	—	—	—	—	—	—
Myco. tuberculosis fraction of adjuvant	1	—	+	—	—	—	—
	2	—	+	—	—	—	—
	3	—	+	—	—	—	—
	4	—	+	+AM	—	—	—
	5	—	—	—	—	—	—
	6	—	—	—	—	—	—
	7	—	+	—	—	—	—
	8	—	+	—	—	—	—

+ signifies presence of granulomatous proliferative lesions.
AM signifies presence of amyloid.

diffuse amyloidosis of varying degree was found in 7 animals. In some sections the deposition of this material was so abundant that only remnants of the normal spleen structure were found (Fig. 3). The amyloid stained with PAS, Gentian violet and Congo red.

TABLE II.—*Summary of Histological Findings in Guinea-pigs*

Material injected	No. of animal	Brain	Liver	Spleen	Kidney	Heart	Lung
Complete adjuvant	1	—	—	+	—	—	—
	2	—	—	—	—	—	—
	3	—	+	+	—	—	Granulomata.
	4	—	+	—	—	—	"
	5	—	—	—	—	—	—
	6	—	—	—	—	—	—
	7	—	—	—	—	—	—
Oil fraction of adjuvant	1	—	—	—	—	—	Granulomata.
	2	—	—	—	—	—	Perivascular infiltration.
Myco. tuberculosis fraction of adjuvant	1	—	+	—	—	—	—
	2	—	+	—	—	—	—
	3	—	+	—	—	—	—

TABLE III.—*Summary of Histological Findings in Hamsters*

Material injected	No. of animal	Brain	Liver	Spleen	Kidney	Heart	Lung
Complete adjuvant	1	—	+	—	—	—	—
	2	+	+	+	—	—	—
	3	—	+	+	—	—	—
	4	—	+	+	—	—	Granuloma.
	5	+	+	+	—	—	Granulomata.
	6	+	+	+	—	—	Granuloma.
	7	+	+	—	—	—	—
Oil fraction of adjuvant	1	—	+	—	—	—	—
	2	—	+	—	—	—	Perivascular infiltration. Granuloma.
	3	+	+	—	—	—	—
	4	—	+	—	—	—	—
	5	+	+	—	—	—	—
	6	—	+	—	—	—	—
	7	+	—	—	—	—	Pneumonia. Giant cell type.
	8	—	+	—	—	—	Perivascular infiltration.
	9	+	+	—	—	—	—
Myco. tuberculosis fraction of adjuvant	1	—	—	—	—	—	—
	2	+	+	—	—	—	Perivascular infiltration.
	3	+	+	—	—	—	—
	4	+	+	—	—	—	—
	5	—	+	—	—	—	Pneumonia.
	6	—	+	—	—	—	—
	7	—	+	—	—	—	—

+ Signifies presence of granulomatous proliferative lesions.

TABLE IV.—*Number of Animals Showing Proliferative Reactions in the Various Organs*

Group	Material injected	Brain			Liver			Spleen			Kidneys			Lungs			Heart		
		M	GP	H	M	GP	H	M	GP	H	M	GP	H	M	GP	H	M	GP	H
I	Complete adjuvant	0 (25)	0 (7)	4 (7)	17 (25)	2 (7)	7 (7)	11 (25)	2 (7)	5 (7)	0 (25)	0 (7)	0 (7)	8 (25)	2 (7)	3 (7)	0 (25)	0 (7)	0 (7)
II	Oil fraction of adjuvant	0 (9)	0 (2)	4 (9)	5 (9)	0 (2)	8 (9)	5 (9)	0 (2)	0 (9)	0 (9)	0 (2)	0 (9)	0 (9)	0 (2)	2 (9)	0 (9)	0 (2)	0 (9)
III	*Myco. tuberculosis fraction of* adjuvant	0 (8)	0 (3)	3 (7)	6 (8)	3 (3)	6 (7)	1 (8)	0 (3)	0 (7)	0 (8)	0 (3)	0 (7)	0 (8)	0 (3)	1 (7)	0 (8)	0 (3)	0 (7)

The total number of animals injected in each instance is shown in parenthesis. M = Mice. GP = Guinea-pigs. H = Hamsters.

Kidneys.—No proliferative lesions were observed. In 4 animals amyloid was found in the glomeruli, in some of these the whole structure being replaced by the deposit (Fig. 4). Chronic pyelonephritis was found in one animal.

Lungs.—In 8 animals scattered granulomatous lesions were found in the parenchyma. Perivascular or peribronchial infiltrations were present, consisting of round cells, histiocytes and occasional giant cells, the latter containing fat droplets which stained with Oil red O. In a few cases foci of acute bronchopneumonia were found.

Group II.—Injected with the oil fraction of the adjuvant

These animals showed marked changes in the liver and spleen only.

Liver.—In 5 animals a proliferation of reticulo-endothelial cells, similar to that found in Group I was noted. However, the intensity of the process was less marked than in Group I and in most of the animals only a mild infiltration was present. In one of the 5 animals diffuse amyloidosis was also observed.

Spleen.—In 5 animals proliferation of the red pulp cells was present and in 3 of them amyloidosis was found. The characteristics of the amyloid as well as the location of the deposits were similar to those found in the animals of Group I.

No changes were found in the *brain*, *kidneys*, *lungs* and *heart*.

Group III.—Injected with the tuberculous fraction of the adjuvant

Liver.—In 6 animals proliferative activity of reticulo-endothelial elements of varying intensity was noted. The characteristics as well as the localization of the lesions were similar to those found in Groups I and II.

Spleen.—In one animal marked proliferation of the red pulp was found. Amyloidosis was also present in this animal.

The *brain*, *kidneys*, *lungs* and *heart* showed no histological changes.

Guinea-pigs

In this species no amyloidosis was found.

Group I.—Injected with complete adjuvant

Reticulo-endothelial cell proliferation in the *liver* was present in 2 animals (Fig. 5); in 2 animals a proliferation of red pulp cells was present in the *spleen*;

the *lungs* of 2 animals showed granulomatous lesions similar to those observed in the mice (Fig. 6). The *brain, heart* and *kidneys* showed no pathological changes.

Group II.—Injected with the oil fraction of the adjuvant

No abnormal findings were noted in any of the organs examined.

Group III.—Injected with the tuberculous fraction of the adjuvant

Proliferation of reticulo-endothelial cells, of varying intensity, was found in the *liver* of all 3 animals. The other organs examined showed no histological changes.

Hamsters

In this species the main changes were found in the brain, liver and lungs. No amyloidosis was observed.

Group I.—Injected with complete adjuvant

Brain.—In 4 animals granulomatous lesions as well as perivascular cuffs were found. Both types of lesion consisted of round cells, histiocytes, occasional plasma cells and occasional polymorphonuclear leucocytes (Fig. 7). No demyelination was found. In some animals the infiltrates extended into the leptomeninges.

Liver.—In the parenchyma and portal spaces in all 7 animals there was marked proliferation of reticulo-endothelial cells in the form of scattered foci or granulomatous lesions.

Spleen.—Red pulp cell proliferation was found in 5 animals.

Lungs.—Granulomatous lesions were present in 3 animals.

Kidneys and heart showed no histological changes.

Group II.— Injected with the oil fraction of the adjuvant

Brain.—In 4 animals lesions such as those seen in Group I were found in varying degree.

Liver.—In 8 animals changes similar to those described in Group I were present.

Lungs.—In 2 animals marked perivascular cuffing was present.

Spleen, kidneys and heart showed no abnormalities.

Group III.—Injected with the tuberculous fraction of the adjuvant

Brain.—Lesions similar to those described in Groups I and II were present in 3 animals.

Liver.—In 6 animals lesions were present similar to those described in the livers of Groups I and II (Fig. 8, 9, 10).

Lungs.—In one animal, perivascular cuffing was found.

In the *spleen, kidneys and heart* nothing abnormal was detected.

DISCUSSION

In experiments published elsewhere it was found that Freund's adjuvant alone, when injected into hamsters, causes perivascular and/or granulomatous lesions in the brain (Tal *et al.*, 1958).

In order to find out whether such inflammatory brain lesions occur in other animals, 2 additional species, namely guinea-pigs and mice, were injected in the

present experiments. No reaction whatever was elicited in the brain in these animals, whereas the brain in the hamsters again showed the lesions previously described.

It was found, furthermore, that the various components of the adjuvant, each when injected separately into hamsters, was capable of eliciting the same type of granulomatous reaction in the brain as that produced by the complete adjuvant. This is supportive evidence for the view that the inflammatory reaction seen in the brain, following the injection of brain substance plus adjuvant into hamsters, is not due to the presence of brain tissue in the injected material, but should be regarded as a non-specific reaction to the injected adjuvant. The presence of brain tissue in the injected material is indispensable only for the production of demyelinative brain lesions in hamsters (Tal *et al.*, 1958).

The injection of the complete adjuvant into mice, guinea-pigs and hamsters in the present investigation elicited a proliferation of reticulo-endothelial elements also in the liver, spleen and lungs, but not in the heart or kidneys (Table IV). When each of the components of the adjuvant was injected alone the result varied according to the organ and species (Table IV). The heart and kidneys again showed no changes ; in the liver, spleen and lungs no reaction at all was observed in some instances but in others proliferative reactions of varying degree were observed.

Amyloidosis was observed, in the present study, in the liver, spleen and kidneys of mice only. When the components of the adjuvant were injected the kidneys showed no amyloid and it was present in the liver only after the injection of the tuberculous fraction. In each instance the proportion of animals showing amyloidosis was lower than after the injection of the complete adjuvant. A similar finding has been reported by Rothbard and Watson (1954) and Gorer (1940) who reported amyloidosis in aged mice. It is relevant to note, therefore, that the mice used in the experiments here reported were 6 weeks old and that the examination of the organs of uninjected control animals revealed no amyloidosis.

SUMMARY

The changes occurring in certain organs of mice, guinea-pigs and hamsters following the injection of Freund's adjuvant or of its components were studied.

Proliferative inflammatory lesions in the brain were observed only in hamsters following the injection of complete adjuvant or of its components. Similar reactions to the complete adjuvant on the part of the reticulo-endothelial system were seen in all 3 species in the liver, spleen and lungs, but not in the heart or kidneys. When each of the components of the adjuvant was injected alone, the result varied according to the organ and species.

Amyloidosis occurred in mice only and was seen in the liver, spleen and kidneys following injection of the complete adjuvant. When the components of the adjuvant were injected the results varied with the component and the organ concerned.

REFERENCES

FREUND, J. AND MCDERMETT, K.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **49**, 548
GORER, P. A.—(1940) *J. Path. Bact.*, **50**, 25.
OLITSKY, P. K. AND TAL, CHLOE.—(1952) *Proc. Soc. exp. Biol., N.Y.*, **79**, 50.
ROTHBARD, S. AND WATSON, R. F.—(1954) *Ibid.*, **85**, 133.
TAL, CHLOE, LAUFER, A. AND BEHAR, A. J.—(1958) *Brit. J. exp. Path.*, **39**, 158.

SOME EFFECTS OF STAPHYLOCOCCAL AND STREPTOCOCCAL TOXINS UPON MAMMALIAN SKIN IN TISSUE CULTURE

J. C. LAWRENCE

From the Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

Received for publication July 29, 1958

CRUICKSHANK and LOWBURY (1953), using a modification of Medawar's (1948) skin culture technique, found that pyocyanin had a toxic action on human skin cells *in vitro*; they suggested that this mechanism might explain the local pathogenic effects of *Pseudomonas pyocyanea* in burns described by Jackson, Lowbury and Topley (1951*a*).

Clinical graft failure attributable to *Streptococcus pyogenes* (Group A) is well known (Jackson, Lowbury and Topley, 1951*b*); similarly *Staphylococcus aureus* may interfere with the healing of burns, though not to the same extent as *Str. pyogenes* (Lowbury, 1957). This finding is supported by the animal experiments of Liedberg, Reiss and Artz (1955), who showed that *Str. pyogenes*, *Ps. pyocyanea* and *Staph. aureus* could all cause failure of skin grafts, *Str. pyogenes* being far more potent in this respect than either of the other 2 organisms.

Little is known of the mechanism by which streptococci and staphylococci damage skin. The present experiments have been carried out to discover whether the haemolysins produced by these bacteria affect skin cultures. The 3-day culture technique used by Cruickshank and Lowbury (1953) has been combined with respiration studies using the differential microrespirometer (Cruickshank, 1954) since a measure of skin metabolism would probably yield more information than the histological method by itself. The value of the microrespirometer in biochemical and metabolic studies of skin has already been demonstrated (*e.g.*, Cruickshank, Trotter and Cooper, 1957; Lawrence and Ricketts, 1957).

MATERIALS AND METHODS

Guinea-pig and human skin were used in this investigation. Guinea-pig skin was cut free-hand from the dorsum of the ear (Cruickshank, 1954); human skin was that surplus to requirement in the grafting of burns.

The standard skin culture medium used throughout was Cruickshank's (1951) modification of Medawar's original medium and consists of: homologous serum 5 parts; Krebs-Ringer phosphate 3 parts; 2 per cent glucose 1 part; and dihydrostreptomycin sulphate, 500 μg./ml., 1 part. Serum was taken with aseptic precautions; the Krebs-Ringer phosphate sterilised by autoclaving and the glucose by boiling for 10 min. The streptomycin was used diluted with sterile physiological saline.

Respiration studies were made with the microrespirometer described by Cruickshank (1954)—this device enables the respiration of small pieces of tissue to be measured accurately over considerable periods of time.

Measurements of oxygen uptake were made for 2 hr. Dilutions of test material were then introduced into the respirometer by means of a Pasteur pipette. Equivalent volumes of saline were added to the controls unless otherwise stated. Respiration measurements were

then continued for a further 6 hr., discontinued overnight (about 15 hr.), recommenced the following day and followed up to 24 hr. after addition of the test substance.

Three-day cultures of skin for histological examination were prepared in the manner described by Cruickshank (1951). These cultures were classified into the 3 types described by Cruickshank and Lowbury (1953): (1) Normal cultures: with migration of epithelium covering the cut surface of the dermis; (2) inhibited cultures: with no epithelial migration but cells staining normally; (3) necrotic cultures: with abnormal staining of epithelial cells and sometimes destruction of the explant.

A special medium was employed to culture bacteria for toxicity tests since many bacterial culture media, nutrient broth in particular, were toxic to skin. Where possible, a non-toxic medium consisting of equal parts of horse serum and 2 per cent glucose in physiological saline was used. The glucose solution was sterilised by Seitz filtration, then mixed with the serum in a sterile container. This medium diluted 1 in 5 had no effect on either skin respiration or 3-day cultures.

Bacterial cultures were sterilised by filtration through sintered glass (porosity No. 5) after removing the bulk of the cells by centrifugation. The sterility of these filtrates was always checked before use.

This method was used to prepare staphylococcal culture filtrates and solutions containing streptolysin S.

Streptolysin O was prepared by the method of Johnson (1955), from streptococci of Group A isolated from burns and septic wounds. Cysteine was used as an activator, and haemolytic activity titrated with 5 per cent rabbit blood. Difficulty was encountered in obtaining streptolysin O from *Str. pyogenes* isolated from burns. These filtrates were almost invariably streptolysin O negative towards rabbit cells (the accepted standard) but some haemolysis of horse cells was observed. Tetracycline therapy had been used on all the burns from which these streptococci had been isolated and possibly they had acquired some of the properties of the abnormal forms described by Lowbury and Hurst (1955*a* and 1955*b*). The Group A streptococci isolated from other sources produced between 30 and 40 minimum haemolytic doses (m.h.d.) per ml. streptolysin O.

Before these haemolysins were tested for effects on skin cells the serum used in the skin culture medium was tested for antibodies to these substances. Guinea-pig serum was usually free from antitoxin but many samples of human sera contained appreciable titres of antitoxin. These samples were rejected.

RESULTS

Staphylococcal α toxin

In preliminary experiments α toxin dissolved in saline was added to respirometer cultures to give a final concentration of 100 m.h.d. per ml. This haemolysin caused striking and consistent changes in respiration. An immediate increase in the respiratory rate was observed, this rose to 25 per cent above the original rate in 30 min. The respiration rate then steadily declined to a very low level 6 hr. after addition of the toxin.

This pattern of behaviour occurred with both human and guinea-pig skin. Fig. 1, line B represents the oxygen uptake of guinea-pig skin exposed to 100 m.h.d. per ml. α toxin which was added at the point indicated by the arrow. Comparable records of a control culture are represented by line A.

A concentration as low as 5 m.h.d. per ml. produced a similar effect but the decrease in respiration was not quite as rapid as it was when 100 m.h.d. per ml. was present in the culture fluid. Amounts of toxin less than 5 m.h.d. per ml. caused detectable changes in respiratory behaviour but in these cases observations had to be extended over a period of 24 hr. As little as 0·2 m.h.d. toxin per ml. caused respiratory stimulation, but this was less than was obtained with larger amounts of toxin, 15 per cent instead of 25 per cent. This small amount of toxin caused 20 per cent respiratory damage 24 hr. after it had been added.

Neutralisation of the α toxin by specific antitoxin

Tests were made to determine the effect of excess antitoxin added to respirometer cultures at different intervals of time after the addition of α toxin. Control experiments showed that antitoxin did not interfere with skin respiration. Previously mixed toxin and antitoxin were also without effect. Antitoxin added 1 min. after the toxin had no immediate effect but 10 per cent respiratory damage was observed after 24 hr. Addition of antitoxin after 15 min. (*i.e.*, almost at the

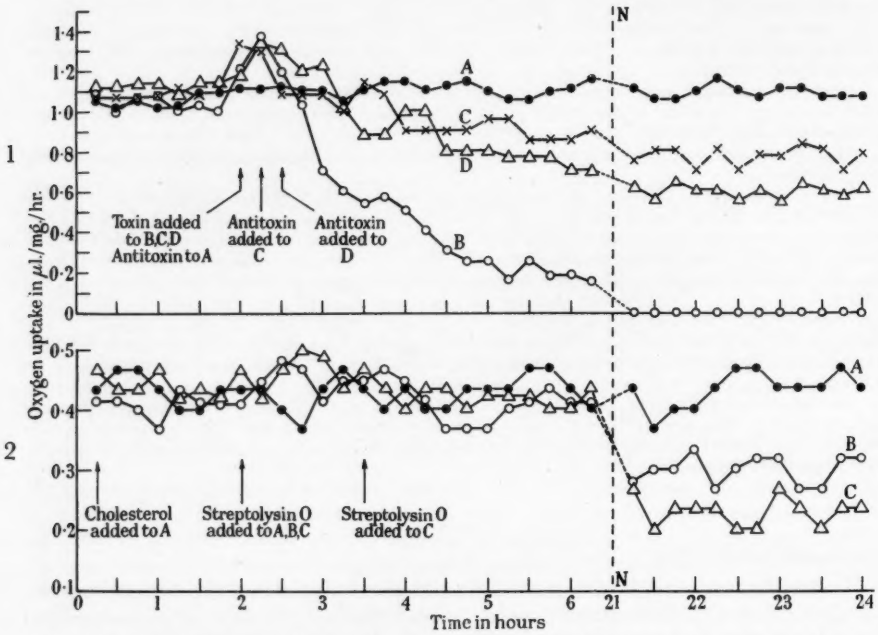


FIG. 1 (upper).—The effect of staphylococcal α toxin on the respiration of guinea-pig skin and the effect of specific antitoxin added at different time intervals. N - - - N overnight period, observations discontinued.

FIG. 2 (lower).—Showing the effect of streptolysin O on the respiration of human skin and the ability of cholesterol to prevent the respiratory changes caused by this toxin.

peak of respiratory stimulation) reduced the respiratory damage to 10 per cent after 6 hr. and to 20 per cent after 24 hr. (see line C, Fig. 1). If the antitoxin was added 30 min. after the toxin greater damage was observed ; 20 per cent decrease in respiration after 6 hr. and 40 per cent after 24 hr. (line D, Fig. 1). Antitoxin added 60 min. after the toxin could not prevent the full amount of damage to the respiratory system caused by the α toxin.

Skin explants cultured for 3 days in the presence of the α toxin were necrotic when examined histologically. Cultures in the presence of antitoxin were normal. Reasonably normal cultures were obtained when antitoxin had been added 15 min. after the toxin, but severe inhibition occurred in cultures to which the antitoxin

had been added 30 min. later; antitoxin added after 60 min. did not prevent the cultures from becoming necrotic.

Staphylococcal culture filtrates

It was found that many bacterial culture media were toxic to skin *in vitro*—nutrient broth diluted 1 in 5 caused 40 per cent decrease in skin respiration after 24 hr. and 3-day cultures were markedly inhibited. Certain bacteria which do not elaborate any toxins, for example *Staphylococcus albus* and streptococci of Group D, eliminated the toxic factor in broth and resultant filtrates had no effect on skin cultures. However, it was not known whether bacterial growth invariably removed this broth " toxin ", so whenever possible the non-toxic medium described earlier was used.

Six strains of *Staph. aureus* (coagulase positive) and 4 strains of *Staph. albus* (coagulase negative) isolated from burns were cultured for various periods of time in the non-toxic medium . Sterile filtrates from these cultures were tested for possible toxicity to skin in the respirometer by making 1 in 5 dilutions with the skin culture medium.

No effects on respiration were observed with staphylococcal culture filtrates from up to 4 days growth of the organisms. With 7-day *Staph. aureus* culture filtrates there was slight stimulation of respiration on addition and a decrease of 10–25 per cent in respiration after 24 hr. This effect could be eliminated with the α antitoxin. These culture filtrates showed haemolytic activity towards rabbit cells and this haemolysis could be neutralised with antitoxin. None of the *Staph. albus* culture filtrates had any effect on skin respiration.

At all stages controls were set up. These were prepared from uninoculated batches of medium incubated for the same time as the bacterial cultures.

α toxin in wound exudate

Cruickshank and Lowbury (1953) demonstrated toxic concentrations of pyocyanin in dressings from burns infected with *Ps. pyocyanea*. An attempt was made to apply this technique to burns infected with coagulase positive strains of *Staph. aureus* to see if the α toxin was present.

Previously weighed pads of gauze were distinctively marked, sterilised and incorporated in a routine dressing. Burns were selected which yielded heavy and virtually pure cultures of *Staph. aureus*. The gauze pad was removed at the next dressing 3–5 days later. It was re-weighed and extracted with a small volume of physiological saline. This extract was centrifuged to remove solid particles and the supernatant solution tested for haemolytic activity against rabbit blood. A control extraction process was carried out using a gauze pad soaked in saline and incubated aseptically for 3 days at 37°.

From 6 dressings and 2 controls no haemolytic activity could be detected. The extract diluted 1 in 5 failed to alter the respiration of skin.

Streptococcal Toxins

Streptolysin O

Sterile streptococcal culture filtrates prepared as described earlier and activated with a little cysteine were tested as 1 in 5 dilutions for effects upon skin respiration. Cysteine was previously shown to have no effect on skin cultures. Control culture

filtrates (uninoculated medium) were slightly toxic, causing a 15 per cent decrease in respiration after 24 hr. Six cultures of Group A streptococci isolated from burns were completely free from toxic effect to skin. As mentioned earlier these streptococci were atypical with respect to production of streptolysin O.

Three strains of Group A streptococci obtained from sources other than burns did produce streptolysin O, and dilutions of these culture filtrates were made with skin culture medium to give haemolysin concentrations between 5 and 8 m.h.d. per ml. Respiratory stimulation occurred on addition of streptolysin O to respirometer cultures. This increase lasted about 1 hr., then the respiratory rate fell back to its original level. The peak of this increase was 15 per cent above the original rate and occurred 30 min. after addition of the haemolysin. No further changes in skin respiration were noted over the next 5 hr. but measurements made on the second day showed a 20 per cent decrease in the respiratory rate. These changes are represented by line B in Fig. 2.

This was a constant finding with both human and guinea-pig skin and could be obtained with as little as 1 m.h.d. streptolysin O.

Oxidised streptolysin O (that is without cysteine) had no effect on skin respiration. Similarly if a little cholesterol was suspended in the skin culture medium the skin was protected from the effects of activated streptolysin O (line A, Fig. 2).

Several samples of streptolysin were dialysed, freeze-dried then reconstituted in one-tenth of their original volume, thus enabling tests to be made with 30–40 m.h.d. per ml. of this haemolysin. This quantity, however, produced no greater changes in respiration than did the smaller amounts.

It was found that a second dose of streptolysin O added 90 min. after the first caused greater respiratory damage after 24 hr. than a single dose. No stimulation of respiration occurred when the second quantity of haemolysin was added. The respiratory damage was about 45 per cent after 24 hr. in this case (line C, Fig. 2).

Three-day skin cultures were found to be quite normal when cultured in up to 40 m.h.d. per ml. streptolysin O. Cultures which had second doses of haemolysin added after the first were inhibited but there was no sign of necrosis.

Streptolysin S

Most of the Group A streptococci isolated produced between 3 and 10 m.h.d. per ml. of this haemolysin when cultured in a medium containing serum. No decrease in haemolytic activity occurred if the filtrates were treated with cholesterol showing that streptolysin O was absent.

A final streptolysin S concentration of 2 m.h.d. per ml. had no effect on skin respiration over a 24 hr. period.

Samples of streptolysin S were dialysed, freeze-dried and incorporated in respirometer cultures at 10 m.h.d. per ml. No immediate respiratory changes were observed but 3 of the 7 samples of guinea-pig skin tested showed a 10 per cent reduction in respiratory rate after 24 hr. None of the 4 samples of human skin tested showed any respiratory changes.

Three-day cultures of skin in concentrations of streptolysin S at 10 m.h.d. per ml. were all normal.

DISCUSSION

These results show that staphylococcal α toxin is extremely toxic to skin but streptolysin O seemed to be somewhat less toxic. Both haemolysins quickly

activate mechanisms causing irreversible damage to skin—in the case of staphylococcal α toxin this was demonstrated by failure of the specific antitoxin added 60 min. after the haemolysin to prevent destruction of the skin cells. Respiration measurements show that damage to skin occurs soon after addition of the toxin, oxygen uptake being greatly reduced in 6 hr. Streptolysin O on the other hand was much slower in causing adverse respiratory changes; these could not be detected for at least 10 hr. However, rapid activation of the toxic mechanism may be inferred from the fact that the potency of streptolysin O is considerably reduced after 2 hr. incubation at 37°.

The mechanism of respiratory stimulation caused by these haemolysins is unknown. Methylene Blue and pyocyanin cause a comparable effect with bacterial cells and it has been postulated that these substances act as oxygen carriers between cytochrome and dehydrogenase systems (Friedheim, 1931). Streptolysin O by virtue of its ability to undergo reversible oxidation might conceivably have a similar effect but it seems unlikely that staphylococcal α toxin could act in this manner.

The microrespirometer seems to be a more sensitive test than the 3-day culture technique. Respiratory damage in excess of 30 per cent after 24 hr. is associated with abnormal histological cultures; with less damage the culture appears normal. The respirometer has the further advantage of yielding information concerning the speed of action of these toxins. It is interesting to note that the typical dermonecrotic toxin—staphylococcal α toxin—causes necrosis of skin *in vitro* whereas streptolysin O which is not recorded as being dermonecrotic (Topley and Wilson, 1955)—only caused inhibition of epithelial migration.

The present experiments suggest that streptolysin S is not toxic to skin; if it is toxic it must be much less potent than either streptolysin O or staphylococcal α toxin.

The role of these haemolysins in burns infected with *Staph. aureus* and *Str. pyogenes* is not clear. Failure of the streptococci isolated from burns to produce much streptolysin O coupled with their high pathogenicity in these wounds (Jackson *et al.*, 1951*b*) suggests that streptolysin O is not of primary importance in the cause of streptococcal graft failure. *Staph. aureus* appears to be of limited pathogenic significance in burns (Lowbury, 1957) yet tissue culture studies show the α toxin to be extremely active against skin. Staphylococcal α toxin could not be demonstrated in the exudate from burns infected with *Staph. aureus* although the organisms present were shown to be potential producers of this toxin. The most likely explanation would appear to be that conditions in burns are unfavourable for production of this toxin. Titration of the antitoxin present in the serum of patients infected with these organisms might throw light on this.

Failure to demonstrate toxic effects other than those attributable to the α haemolysin from the staphylococcal culture filtrates suggests that other toxins are not important in the infection of burns by these organisms. The β toxin is not dermonecrotic (van Heyningen, 1950) so might possibly be without effect. The γ and δ toxins require a high CO_2 content and a low pH respectively for production; neither of these conditions are likely to be achieved in an infected burn. The finding that relatively old culture filtrates of *Staph. aureus* were toxic is in keeping with the earlier observation that this toxin develops in culture after some days (Neisser and Wechsberg, 1901).

The part played by hyaluronidase and fibrinolysins in the infection of burns

is also not clear. It was found that all the streptococci produced streptokinase and some of the staphylococci formed hyaluronidase but testicular hyaluronidase and streptokinase appear to have no ill-effects upon skin *in vitro* (Lawrence, 1958).

Recently it has been suggested that dermonecrosis caused by staphylococcal α toxin is due to vaso-constriction (North and Doery, 1958) but the current investigation clearly shows that skin cells themselves are killed by this toxin.

SUMMARY

The effects of staphylococcal α toxin, streptolysin O and streptolysin S on skin respiration and on the migration and viability of epithelial cells *in vitro* were investigated.

Staphylococcal α toxin was found to be extremely toxic to skin, streptolysin O was less so. Streptolysin S seemed to be comparatively non-toxic to skin cultures.

Both staphylococcal α toxin and streptolysin O appeared to cause rapid activation of toxic mechanisms in skin. Specific staphylococcal anti-toxin was not able to prevent the effect of the α haemolysin if added 1 hr. later, but afforded partial protection if added before this time. Previously mixed toxin and antitoxin had no effect on skin cultures.

These results were briefly discussed in relation to the infection of burns by *Staph. aureus* and *Str. pyogenes* Group A. It was concluded that streptolysin O was not of primary importance in the cause of streptococcal graft failure and it was suggested that conditions found in burns were not suitable for production of staphylococcal toxin.

I should like to thank Dr. J. P. Bull and Dr. E. J. L. Lowbury for much valuable advice and criticism. The staphylococcal α toxin and specific antitoxin was obtained from Miss Mollie Barr of The Wellcome Research Foundation.

REFERENCES

Cruickshank, C. N. D.—(1951) *Nature*, **168**, 206.—(1954) *Exp. Cell. Res.*, **7**, 374.

Idem and Lowbury, E. J. L.—(1953) *Brit. J. exp. Path.*, **34**, 40.

Idem, Trotter, M. D. and Cooper, J. R.—(1957) *Biochem. J.*, **66**, 285.

Friedheim, E. A. H.—(1931) *J. exp. Med.*, **54**, 207.

Jackson, D. M., Lowbury, E. J. L. and Topley, E.—(1951*a*) *Lancet* ii,, 137.—(1951*b*) *Ibid.*, ii, 705.

Johnson, G. D.—(1955) *J. clin. Path.*, **8**, 296.

Lawrence, J. C.—(1958) Ph.D. Thesis, University of Birmingham.

Idem and Ricketts, C. R.—(1957) *Exp. Cell Res.*, **12**, 633.

Liedberg, N. C-F., Reiss, E. and Artz, C. P.—(1955) *Ann. Surg.*, **142**, 92.

Lowbury, E. J. L.—(1957) *Lancet*, ii, 305.

Idem and Hurst, L.—(1956*a*) *J. clin. Path.*, **9**, 59.—(1956*b*) *J. gen. Microbiol.*, **15**, 324.

Medawar, P. B.—(1948) *Quart. J. microscop. Sci.*, **89**, 187.

Neisser, M. and Wechsberg, F.—(1901) *Z. Hyg. Infekt. Kr.*, **36**, 299.

North, E. A. and Doery, H. M.—(1958) *Nature*, **181**, 1542.

Topley and Wilson.—(1955) 'Principles of Bacteriology and Immunity'. London (Arnold).

van Heyningen, W. E.—(1950) 'Bacterial Toxins'. Oxford (Blackwell Scientific Publications).

THE IMMUNOLOGICAL RESPONSE OF MARROW TREATED X-IRRADIATED RABBITS TO BOVINE GAMMA-GLOBULIN

D. HOBSON,* K. A. PORTER AND J. L. WHITBY

From the Wright-Fleming Institute and Department of Pathology, St. Mary's Hospital Medical School, London, W.2

Received for publication August 5, 1958

ANIMALS exposed to massive doses of total body X-irradiation are known to have a greatly impaired capacity to respond immunologically to antigens experienced for the first time in the post-irradiation period (Dixon, Talmage and Maurer, 1952). This depression of function varies with the dose of irradiation but may persist for more than 3 weeks after exposure (Taliaferro, Taliaferro and Janssen, 1952).

It is also known that following an otherwise lethal dose of X-rays a high percentage of animals will survive the acute irradiation damage if treated by an intravenous injection of bone marrow. This is due to repopulation of the depleted host haemopoietic tissues by the injected cells and their progeny leading to rapid haematological recovery and prevention of death from infection and haemorrhage (Ford, Hamerton, Barnes and Loutit, 1956 ; Porter, 1957). The fate of lymphoid tissue in the marrow treated animal is less certain. The possibilities are that the lymphoid tissue is of donor origin (Ford *et al.*, 1956 ; Mitchison, 1956), or is of host origin due to post-irradiation regeneration (Congdon, Makinodan and Gengozian, 1957), or that no lymphoid tissue is either donated or regenerated.

The answer to this question may be of particular importance in explaining the limited survival of animals given homologous marrow as opposed to isologous marrow. If the lymphoid tissue is derived from the donor an immune response may develop against host antigens (Trentin, 1956), if it is of host origin an immune response may develop against the marrow homotransplant (Congdon *et al.*, 1957) and if antibody-forming cells are neither regenerated by the host nor transferred from the donor, the irradiated animal will be left without any specific defence against microbial invasion.

Landsteiner and Chase (1942) have established that cells capable of producing antibody can be transferred from lymph nodes of immunized animals to normal homologous recipients. Roberts and Dixon (1955), in such animals with transplanted lymphoid tissue, have shown that elimination of homologous antigen is faster than in normal animals, that is, their response to injected antigen is comparable with that of immune animals as described by Glenny and Hopkins (1923) and Talmage, Dixon, Bukantz and Dammin (1951).

These findings have been utilized in the present experiments which were undertaken to determine whether antibody-forming cells are present after marrow

* Present address : Virus Research Laboratory, Lodge Moor Hospital, Sheffield 10.

treatment of X-irradiated animals, and if so whether they are of donor or host origin.

MATERIALS AND METHODS

Animals.—Young adult Chinchilla rabbits, which were not inbred in the genetic sense, weighing 2·0–3·0 kg. were used throughout. The diet was No. 18 of Bruce and Parkes (1946) supplemented by hay, carrots, apple, cabbage, bran, oats and iodine.

X-irradiation.—Total body X-irradiation was administered as a horizontal beam to 2 animals at a time from a Westinghouse machine under the following conditions : 220 kV 12·5 mA., 70 cm. target to skin distance, 1·0 mm. Cu and 1·0 mm. Al filters, H.V.L. 1·8 mm. Cu. dose rate to skin 33·4 r per min., to centre of animal 21·1 r per min. The dosage was divided : an initial dose of 600 r at the centre of the animal was followed 24 hr. later by an additional 500 r.

Immunization of animals.—Female rabbits were immunized with bovine γ-globulin (BGG) given intravenously in doses progressing from 0·1 to 2·0 mg.; the antigen employed was Cohn fraction II of bovine plasma proteins (Armour batch No. 23929) which contains approximately 95 per cent γ-globulin. The material was freshly dissolved in saline containing 1 in 10,000 merthiolate (Lilly) for each day's injections.

In general, circulating antibody after this course of injections was of short duration. The immunized female rabbits were used as donors of bone marrow 10–15 days after the last dose of BGG.

Bone marrow preparation.—Marrow was collected under sterile conditions from the bones of all 4 limbs of female rabbits, suspended in saline, and the volume adjusted so that each irradiated male rabbit received approximately 1200 ($\times 10^6$) nucleated cells. This was administered as a single intravenous injection into the ear 1–2 hr. after the last dose of irradiation.

Antibiotic therapy.—All irradiated rabbits were given 70–75 mg. of tetracycline hydrochloride per day for 3 weeks post-irradiation (Porter and Murray, 1958).

Haematology.—Haemopoietic recovery was assessed on the results of total and differential leucocyte counts, microhaematocrit and haemoglobin estimations, and examination of blood films at 2-day intervals. Successful marrow transplantation was assumed if a minimum of 6 granulocytes showing nuclear " drumsticks " typical of the female could be found in the stained film (Porter, 1957).

The fate of bovine γ-globulin (BGG) injected into marrow treated rabbits.—The rate of removal of the heterologous protein from the blood stream after a single intravenous injection was followed by the use of isotope-labelled material. BGG (Armour batch No. 23929), combined with ^{131}I was kindly supplied by Dr. A. S. MacFarlane of the National Institute for Medical Research.

Male rabbits received a single intravenous injection of BGG dissolved in merthiolate saline in a dosage of 10 mg./kg. body weight containing approximately 10 μc./kg. of radioactive material.

One hour after injection and at 2-day intervals thereafter, until the death of the animal or for a minimum of 16 days, small heparinized blood samples were taken for determination of plasma radioactivity.

The estimation of circulating antigen.—Filter paper disks 2·5 cm. in diameter were glued to metal planchettes and impregnated with 0·25 ml. samples of plasma and dried under a radiant heat lamp. An aliquot of each plasma sample was mixed with an equal volume of 20 per cent trichloracetic acid, and the protein free supernatant obtained by centrifugation ; 0·5 ml. volumes (equivalent to 0·25 ml. plasma) were pipetted on to similar paper disks on planchettes.

The radioactivity of the samples was determined with a Geiger-Muller end-window tube in a Chicago nuclear scaling apparatus. The protein-bound radioactivity (expressed as counts per minute per 0·25 ml. plasma) was corrected for background and decay, expressed as a percentage of the initial count and plotted graphically on a $\log_{10}$ scale against time (in days) after injection.

The measurement of antibody production.—Antibody production was measured indirectly by the capacity of serum to precipitate BGG ^{131}I as compared to hyperimmune and normal rabbit sera. The method used was based on that of Eisen and Keston (1949) as modified by Talmage and Maurer (1953).

EXPERIMENTAL

In these experiments 72 normal male rabbits who had had no previous experience of bovine γ-globulin (BGG) were used. All were challenged with a standard dose of BGG ^{131}I.

Of the 72 animals 60 were exposed to whole body X-irradiation, and each animal was allotted to one of 3 groups :

Group 1 was a control group of 11 animals not given bone marrow who received BGG ^{131}I 1–2 hr. after final irradiation.

Group 2 consisted of 14 rabbits treated with bone marrow from normal donors; 8 were injected with BGG ^{131}I 10 days after irradiation and six 1 hr. after irradiation.

Group 3 consisted of 41 rabbits treated with homologous bone marrow from donors previously immunized against BGG. A single injection of BGG ^{131}I was given within 1 hr. of marrow treatment to 11 of these animals and 10 days later to the other 30 rabbits.

Group 4.—A fourth group of 12 rabbits was not irradiated, of these 4 were given marrow from normal donors, 4 were given marrow from donors previously immunized against BGG and 4 received no marrow at all.

All were given BGG ^{131}I 1 hr. after marrow where it was given.

Group 5.—A final group consisted of 11 animals with varying previous experience of BGG. Some of these rabbits were survivors from the above experimental groups. All were given a second standard dose of BGG ^{131}I.

RESULTS

Group 1 (*irradiated controls*)

During the first 9 days after injection of BGG ^{131}I the plasma-bound radioactivity counts showed a closely similar trend in all animals (Fig. 1), the antigen being removed from the circulation at a constant slow rate, with an average disappearance rate (ADR) of 29·3 per cent per day. Only 2 rabbits survived to the end of the period of observation : one (L10) showed evidence of an immune response in that the ADR increased to 43 per cent from the 9th day and a low titre of circulating antibody developed.

Group 2 (*irradiated animals treated with normal marrow*)

This group was given BGG ^{131}I 10 days after marrow treatment. Five animals died within the ensuing 5 days but during that period eliminated antigen slowly. The other 3 animals eliminated antigen at a uniformly slow rate (ADR 28) for the remaining period and at no time showed an accelerated rate of clearance (Fig. 2). The 6 rabbits given BGG ^{131}I 1 hr. after marrow treatment also showed no evidence of any specific immune response to the injected antigen (maximum ADR 35).

Group 3 (*irradiated animals treated with immune marrow*)

Given BGG ^{131}I 1 hr. after immune marrow treatment.—The distribution of percentage fall of plasma protein-bound radioactivity counts in this group is shown in Fig. 3. During the first 4 days, during which 1 rabbit died, antigen was removed

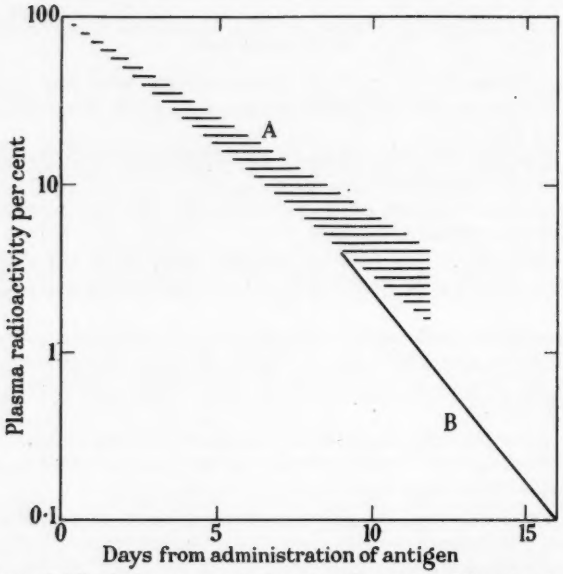


FIG. 1.—The rate of fall of plasma protein bound radioactivity following an injection of BGG ^{131}I in rabbits 1 hr. after X-irradiation. A represents rabbits L2, 4, 6, 8, 12, 16 and 18. B represents rabbit L10.

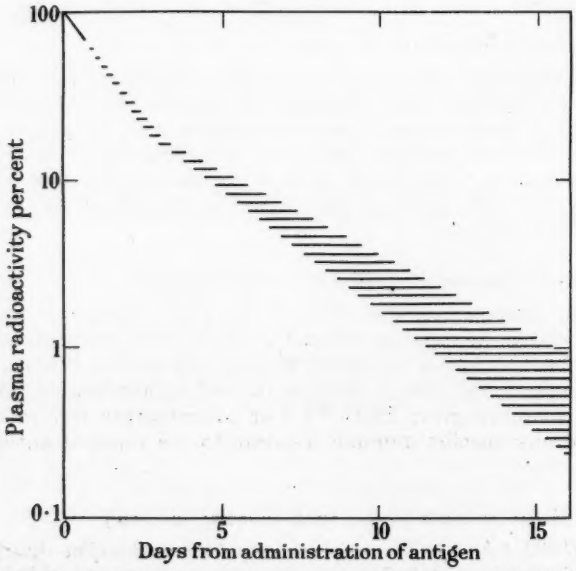


FIG. 2.—The rate of fall of plasma protein bound radioactivity in X-irradiated rabbits following an injection of BGG ^{131}I 10 days or 1 hr. after marrow transfusion from normal animals.

at similar slow rates in all animals and the mean ADR of 36·5 per cent per day was little greater than in irradiated rabbits not receiving marrow treatment.

Thereafter 4 animals with no cytological evidence of successful marrow homotransplantation eliminated antigen slowly. Two of these (L1 and L3) subsequently regenerated their own marrow and survived and during this period showed a slightly increased rate of elimination (ADR 34 per cent) with maximum daily values of 38 per cent and 49 per cent respectively. Both rabbits were subse-

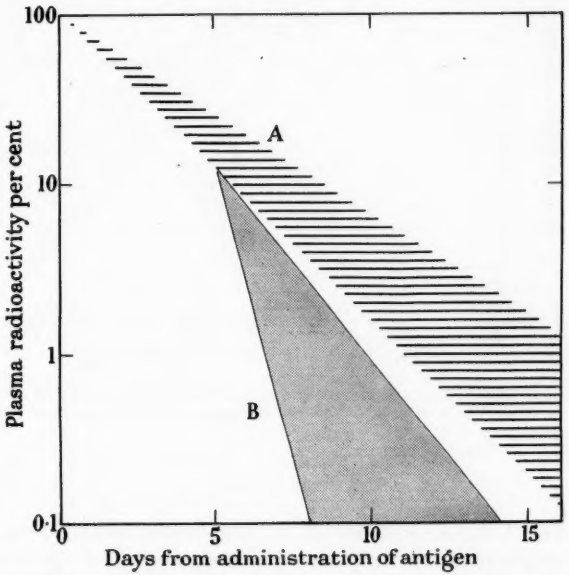


FIG. 3.—The rate of fall of plasma protein bound radioactivity in X-irradiated rabbits following an injection of BGG ^{131}I 1 hr. after marrow transfusion from immune animals. A represents rabbits L1, 3, 9 and 17. B represents rabbits L5, 7, 19, 21 and 23.

quently found to have small amounts of circulating antibody during the 2 weeks following antigen disappearance.

The remaining 5 rabbits with successful marrow homotransplants as shown by the appearance of female granulocytes in the peripheral blood, showed an abrupt rise in antigen clearance after the 4th day, with maximum levels of 53·8 per cent in rabbit L7, 62·2 per cent in L5, 70·2 per cent in L21, 74·3 per cent in L23 and 82·4 per cent in L19; the 99 per cent antigen elimination level was attained between the 7th and 8th day after injection. In 4 ultimate survivors specific antibody was demonstrated (Table). The antigen-precipitating capacity of L19, L21 and L23 serum was comparable with that of repeatedly injected normal rabbits; the antibody was of higher titre and of greater duration than in normal animals similarly injected with a single dose of antigen.

Given BGG ^{131}I 10 days after immune marrow treatment.—The rate of elimination of BGG ^{131}I in this group is shown in Fig. 4. All but 5 rabbits died within 16

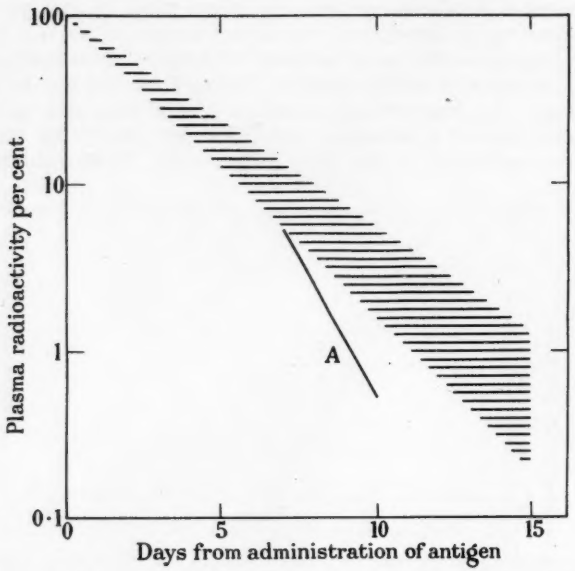


FIG. 4.—The rate of fall of plasma protein bound radioactivity in X-irradiated rabbits following an injection of BGG ^{131}I 10 days after marrow transfusion from immune animals. A represents rabbit P1.

TABLE.—*Relative Antibody Production in Non-irradiated and Irradiated Animals With and Without Marrow Homografts, as Assessed by Percentage Precipitation of a Quantity of Antigen* 85 *per cent of which was Precipitated by a Hyperimmune Rabbit Serum*

Rabbit No.	Experimental procedure	Experience of bovine γ-globulin	Mean percentage of antigen precipitated by serum at various intervals after disappearance of circulating antigen			
			3 days	8 days	2 weeks	3 weeks
L 19	Irradiated rabbits, treated with marrow from immunized donors	10 mg./kg. BGG ^{131}I 1 hr. after marrow transplantation	92·0	86·9	84·5	81·2
L 21			81·0	74·8	70·2	68·0
L 23			76·4	71·6	63·0	59·2
L 1			34·6	28·4	11·1	4·0
L 3			34·7	31·0	23·4	Dead
L 7			44·5	52·3	20·0	„
RSH Sal	Normal rabbits	Hyperimmunized with BGG	80·7	86·0	70·9	36·8
			85·6	85·4	82·7	87·0
L 18	Irradiated rabbits, not treated with bone marrow	10 mg./kg. BGG ^{131}I 1 hr. after irradiation	2·7	1·9	Dead	—
L 10			25·6	9·5	1·4	—
M4	Normal	10 mg./kg. BGG ^{131}I	22	10·3	4·8	—

days of the antigen injection. However, the fall of radioactivity counts in the 13 survivors beyond 7 days was similar ; antigen clearance was slow (ADR 34·4) and there was no augmentation in clearance rate after the 4th day. The 99 per cent antigen elimination level had not been reached before the 15th day, and in neither of 2 ultimate survivors P11 and P12 was circulating antibody demonstrable. In the rabbit P1 which survived for 10 days after injection the ADR was 38 per cent with a maximum rate of 50 per cent which was higher than in any other animal in the group, and the 99 per cent antigen elimination level had been reached between the 8th and 9th day after injection.

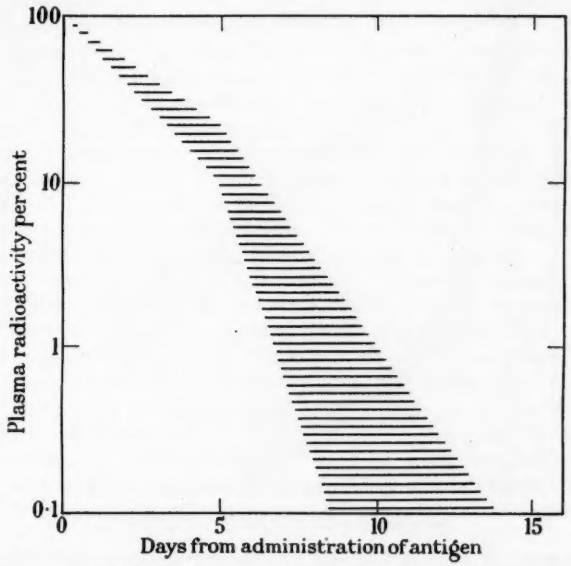


FIG. 5.—The rate of fall of plasma protein bound radioactivity following an injection of BGG ^{131}I in normal rabbits.

Group 4 (*non-irradiated animals*)

In the marrow treated animals within this group there was no cytological evidence of establishment of the donor tissue, female granulocytes failing to appear in the peripheral blood at any time. The response to antigen of the whole group is shown in Fig. 5.

The removal of circulating antigen over the first 4 days was only slightly faster than in irradiated rabbits, with a mean ADR of 35·4 per cent per day. Thereafter, the rate of antigen disappearance changed abruptly, rising to a maximum of 71·7 per cent between the 4th and 6th day ; in all animals the 99 per cent elimination level of antigen was reached between the 7th and 8th days after injection. Circulating antibody appeared in all cases after antigen disappearance (Table) but the apparent concentration was low, and no detectable antibody remained by the 3rd week after its first appearance.

Group 5 (response to a second antigen injection)

The 11 animals in this group fell into 2 subgroups. In one antigen elimination was rapid (A11, A12, C7, C8, L19, 21, 23, M4). Of these A11 and 12 and C7 and 8 were normal rabbits hyperimmune to BGG following multiple injections while M4 had received only one previous injection. L19, 21 and 23 were survivors of Group 3 who had evidence of a successful marrow homograft. In all these animals antigen elimination was rapid (ADR 98–99) except M4 which was slightly less and circulating antibody subsequently appeared rapidly and to high titre (Fig. 6).

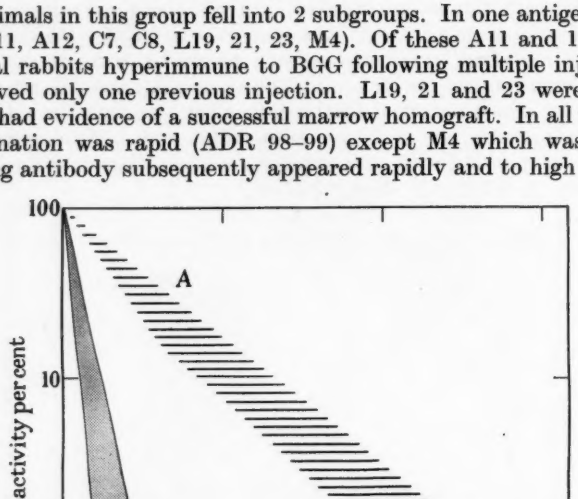


FIG. 6.—The rate of fall of plasma protein bound radioactivity in irradiated and marrow treated rabbits with previous experience of BGG, following a second injection of BGG ^{131}I. A represents rabbits K10, L1 and 18. B represents rabbits A11, 12, C7, 8, L19, 21, 23 and M4.

The other rabbits all eliminated antigen slowly. L1, which had regenerated its own bone marrow, had previously been given antigen after irradiation and marrow treatment; there was some evidence of immune response at that time but antibody production had been weak and transient. Rabbits L18 and K10 had previously been irradiated but not given bone marrow; neither had shown an immune response to the first injection of antigen. In none of these 3 animals (Fig. 6) did the ADR increase above 35 per cent on the 4th or subsequent days after the second antigen injection. The 99 per cent antigen elimination level was not attained until the 13th day after injection, and no antibody was subsequently found.

DISCUSSION

These experiments provide no conclusive answer to the question of the source of the lymphoid tissue in survivors of X-irradiation treated with bone marrow,

but it is clear that immediately after marrow treatment living immune cells of donor origin are present in the recipient and are functionally active against their specific antigen. Ten days later immunological activity could no longer be demonstrated. The data is thus similar to that of Sterzl (1955) and Mitchison (1956) who showed in rabbits and mice respectively that spleen cells from actively immune animals continued to form demonstrable amounts of antibody for at least 20 days after transference to irradiated animals. Mitchison was also able to detect antibody, in control non-irradiated animals similarly treated, at lower titre over the same period, in both isologous and homologous mouse strains.

It is possible that so few cells immune to BGG were injected in relation to the ultimate cell population of the lymphoid tissue, that they were no longer capable of giving a characteristic immune response following the injection of antigen. Another possibility is that until the lymphoid tissue has really become re-established its cells may be incapable of satisfactory immune behaviour and Gengozian and Makinodan (1958) have shown that following the injection of antigen in mice recovering from X-irradiation, responsiveness is depressed for at least 45 days and is minimal up to 5 days. It is therefore desirable that similar experiments should be performed in animals when their lymphoid tissue has recovered normal activity. However, there is a difficulty in that a delayed mortality is frequently encountered which hinders the collection of groups of animals.

Our work has been complicated by an apparent heavy mortality in the rabbits which received BGG 10 days after irradiation and initially it was suspected that the administration of antigen to these animals might have been a precipitating cause of death or that animals with immune lymphoid tissue were dying and only non-immune animals surviving. However, it seems likely that the bulk of the mortality was due to the fact that animals had to survive for a period of 26 days if they were to live 16 days after the administration of antigen. Neither the overall survival to 26 days, nor the mortality 10–15 days after irradiation were significantly greater than in other groups. The survival figures are much lower than those of Porter and Murray (1958) who obtained 62 per cent survival for 4 weeks and 40 per cent survivors for at least 32 weeks. Our heavy mortality can probably be accounted for by the repeated bleedings and manipulations necessary to obtain samples at the early and critical post-irradiation period.

The study of antigen removal in the few animals surviving long enough to receive antigen for the second time does point to the derivation of lymphoid cells from the marrow homograft. In these animals (L19, 21 and 23) not only was there a high level of precipitating antibody after elimination of the first dose of BGG ^{131}I but the second challenge dose was removed in a manner typical of hyperimmune rabbits such as could reasonably be expected only if the antibody forming cells had been derived from an immune animal. In the animal L18 which received no marrow treatment elimination of the second dose of antigen was no more rapid than elimination of the first, and L1 which received immune marrow but never showed the presence of circulating female granulocytes also eliminated the second dose of antigen slowly.

SUMMARY

Rabbits were exposed to whole body X-irradiation and treated with homologous bone marrow. The donor animals had been previously immunized with bovine γ-globulin. Recipients were challenged with radio-iodinated bovine γ-globulin

1 hr. or 10 days after marrow treatment. At 1 hr. antigen was eliminated in the manner of immune animals. At 10 days no increased rate of removal could be demonstrated. Surviving rabbits were given a second dose of antigen 5 weeks later; those with successful marrow transplants acted as immune animals.

We are indebted to Dr. A. S. MacFarlane, National Institute for Medical Research, Mill Hill, for kindly preparing the BGG ^{131}I; to Dr. M. Hulbert and Sister Woodward, Radiotherapy Department, St. Mary's Hospital, for providing irradiation facilities; to Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York for generous supplies of tetracycline hydrochloride; and to Miss Jane Rendall for valuable technical assistance.

This work was supported in part by a grant from the Medical Research Council to one of us (K. A. P.) for which we are most grateful.

REFERENCES

Bruce, H. M. and Parkes, A. S.—(1946) *J. Hyg., Camb.*, **44**, 501.
Congdon, C. C., Makinodan, T. and Gengozian, N.—(1957) *J. Nat. Cancer Inst.*, **18**, 603.
Dixon, F. S., Talmage, D. W. and Maurer, P. H.—(1952) *J. Immunol.*, **68**, 693.
Eisen, H. N. and Keston, A. S.—(1949) *Ibid.*, **63**, 71.
Ford, C. E., Hamerton, J. L., Barnes, D. W. H. and Loutit, J. F.—(1956) *Nature*, **177**, 452.
Gengozian, N. and Makinodan, T.—(1958) *J. Immunol.*, **80**, 189.
Glenny, A. T. and Hopkins, B. E.—(1923) *J. Hyg., Camb.*, **21**, 142.
Landsteiner, K. and Chase, M. W.—(1942) *Proc. Soc. exp. Biol. N.Y.*, **49**, 688.
Mitchison, M. A.—(1956) *Brit. J. exp. Path.*, **37**, 239.
Porter, K. A.—(1957) *Ibid.*, **38**, 401.
Idem and Murray, J. E.—(1958) *J. Nat. Cancer Inst.*, **20**, 189.
Roberts, J. C. and Dixon, F. J.—(1955) *J. exp. Med.*, **102**, 379.
Šterzl, J.—(1955) *Folia Biologica*, **1**, 193.
Taliaferro, W. H., Taliaferro, L. G. and Janssen, E. F.—(1952) *J. infect. Dis.*, **91**, 105.
Talmage, D. W. and Maurer, P. H.—(1953) *Ibid.*, **92**, 288.
Idem, Dixon, F. J., Bukantz, S. C. and Dammin, G. J.—(1951) *J. Immunol.*, **67**, 243.
Trentin, J. J.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **93**, 98.

VASCULAR PATTERN IN THE INDUCED PRIMARY CARCINOMA OF THE LIVER OF RATS

A. S. KRISHNA MURTHY

From the Indian Cancer Research Centre, Parel, Bombay, India

Received for publication August 7, 1958

CHANGES in the vascular pattern of the liver in cirrhosis were first reported by Herrick (1907) and McIndoe (1928). Since then various techniques have been tried to study the gross and microscopic anatomy of the hepatic vascular bed. These studies have helped in understanding the functional anatomy of the liver and also the histogenesis of cirrhosis.

The pattern of blood flow in neoplasms of the liver had received scant attention except for the work done by Breedis and Young (1954) and by Mann, Wakim and Baggenstoss (1953). These workers carried out their studies mainly on established tumours. It was therefore decided to observe the progressive changes in the vascular pattern of the liver of rats fed with the azo dye, butter yellow (*p*-dimethylaminoazobenzene), where the changes preceding and accompanying tumour formation could be studied.

MATERIALS AND METHODS

For this study 69 rats of the Wistar strain of both sexes, 8–10 weeks of age, were used. They were fed, *ad libitum*, with a diet of the following composition : Rice powder (unpolished) 80 per cent, casein 15 per cent, shark liver oil 1 per cent, salt 2 per cent, olive oil 2 per cent, butter yellow 0·06 per cent. The azo dye in an olive oil suspension was thoroughly mixed with the diet.

Under ether anaesthesia, the peritoneal cavity was opened and the portal, splenic and mesenteric veins were freed from their membranous coverings. A dilute solution of Indian ink in water was perfused through the superior mesenteric vein just below the pancreas as slowly as possible. After perfusion was complete, the thoracic and abdominal vena cavae were ligated. The liver was removed *in toto* and fixed in 10 per cent formalin. Frozen sections were cut at a thickness of 75 μ for the study of vascular pattern. A few sections were counterstained by Ehrlich's haematoxylin.

Paraffin sections were cut at a uniform thickness of 5 μ, and were stained by Ehrlich's haematoxylin and aqueous eosin, Picro-Mallory for the demonstration of connective tissue and periodic acid Schiff (PAS) for the demonstration of mucin and glycogen. Sections stained by PAS were counterstained with Celestin blue and Mayer's haematoxylin ; later they were rinsed in a saturated solution of picric acid in water, followed by dehydration and clearing. In order to distinguish glycogen from other PAS positive substances a few control sections were subjected to salivary digestion before staining.

Methods in use for the study of the vascular pattern of the liver are, transillumination by quartz rod microscopy, portal venography, the corrosion-cast method and the injection method.

The transillumination method was used by Wakim and Mann (1942), Knisely (1948) and Seneviratne (1949). It consists in observing the circulation of blood in the liver under a microscope using slit quartz rod illumination. This method is by far the best and free from artefacts. Portal venography was used by Daniel, Prichard and Reynell (1952) and is of help in establishing the presence of collateral circulation and studying the vascular bed in

general, but the finer pattern cannot be studied. The corrosion-cast method has been in wide use, it gives a general idea of the vasculature, but it is doubtful whether a complete picture can be reconstructed.

The injection method enjoys great popularity on account of its simplicity and finer details of the vascular pattern can be studied. Nevertheless this method has a few shortcomings. The pressure at which the dye is injected is not the same as that within the vessel ; it is difficult to perfuse a dye through the hepatic artery because of its small calibre and perfusion through the hepatic vein will be at a retrograde pressure. After due consideration it was felt that the advantages of the method outweighed the disadvantages.

RESULTS

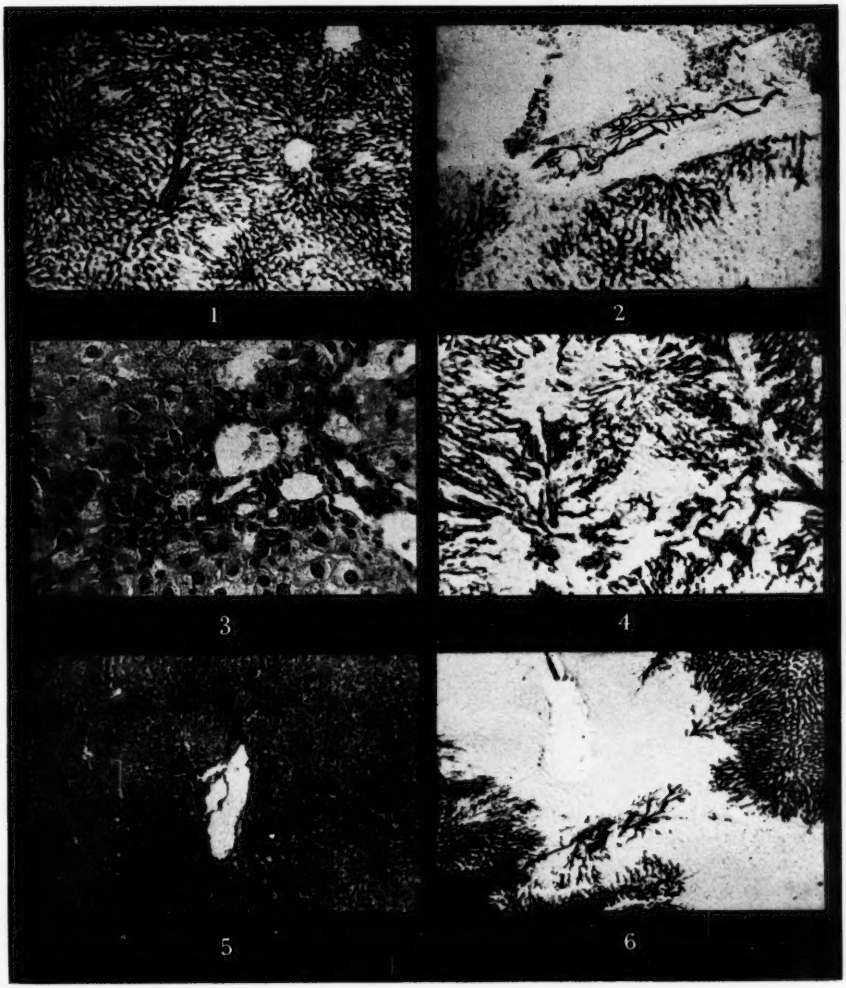
Vascular Pattern of the Normal Liver

A lot of work on the vascular pattern of the liver has been done recently. Elias (1953) and Elias and Popper (1955) demonstrated the arborization of the portal vein in the liver and its significance in understanding the early lesions in cirrhosis. Hence only the salient features of the vascular pattern are described here.

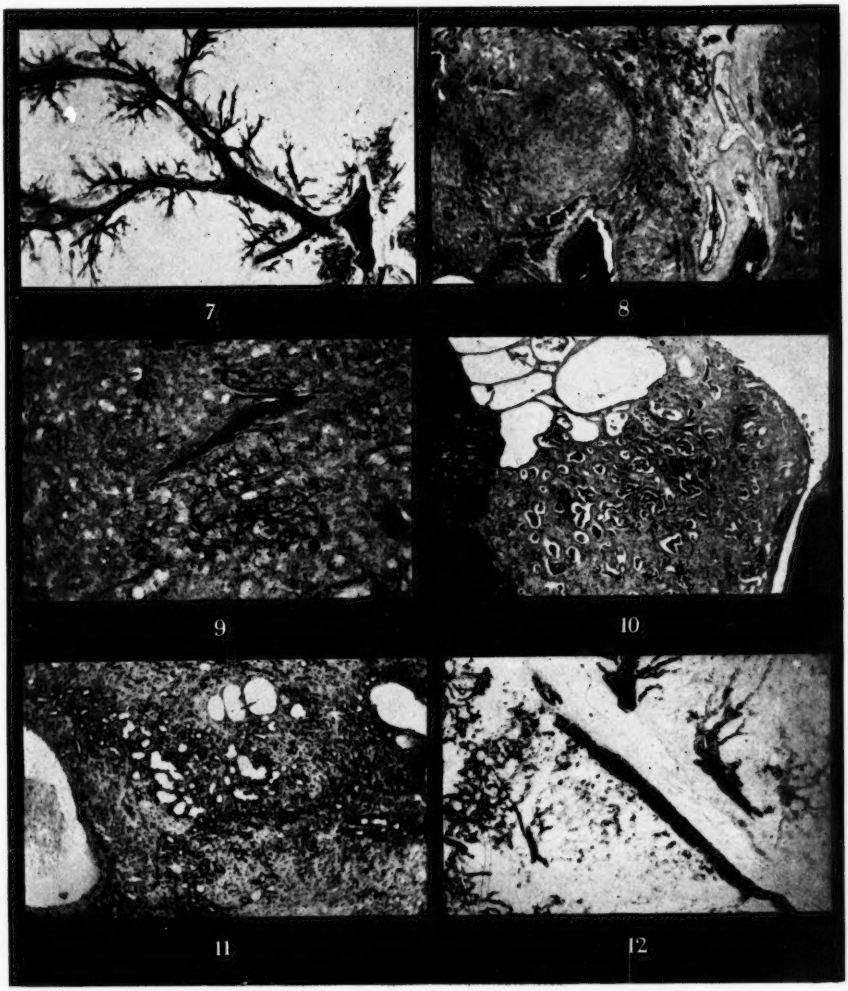
No lobular pattern could be made out in sections of the normal liver perfused with Indian ink through the portal vein, but there was a definite and distinct relationship between the big vessels. The arborization of the portal vein was recognized. The conducting and the marginal distributing veins were recognized after studying a number of sections. A plexus of small vessels filled with Indian ink was observed in a few portal triads, this may be the peribiliary plexus (Andrews, Maegraith and Wenyon, 1949) (Fig. 1 and 2).

EXPLANATION OF PLATES

FIG. 1.—Vascular pattern of the liver of a normal rat. Indian ink injected into the portal vein. × 72.
FIG. 2.—" Peribiliary plexus." × 50.
FIG. 3.—Liver at 30 days. Note the inflammatory cells in the portal triad ; parenchymatous cells with granular cytoplasm and some pyknotic nuclei. H. and E. × 202.
FIG. 4.—Liver at 60 days. Note the bloated sinusoids and uneven filling of the dye. × 72.
FIG. 5.—Liver at 60 days. Moderate increase of connective tissue in the periportal zone. H. and E. × 72.
FIG. 6.—Liver at 90 days. Only the sinusoids around some of the big vessels filled with the dye. × 72.
FIG. 7.—Liver at 120 days. A conducting vein with its main branches filled with the dye. × 72.
FIG. 8.—Liver at 120 days. Connective tissue septum between two pseudolobules. H. and E. × 72.
FIG. 9.—Liver at 150 days. A vessel and a few sinusoids entrapped in a cholangiofibrosis. × 72.
FIG. 10.—Cholangiofibrosis. Lumen of bile ducts contain mucin. PAS. × 50.
FIG. 11.—Liver at 180 days. Proliferation of interlobular bile ducts. H. and E. × 72.
FIG. 12.—Liver at 210 days. A collateral vein running along the connective tissue septum. × 50.
FIG. 13.—Liver at 210 days. Carmine injected into the hepatic vein in the vascular spaces of the tumour. × 72.
FIG. 14.—Liver at 240 days. Approximation of big vessels and their tortuosity suggestive of collapse of parenchyma. × 50.
FIG. 15.—Liver at 270 days. Absence of dye (injected into the portal vein) in the vascular spaces of the tumour (indicated by arrow). × 72.
FIG. 16.—Liver cell carcinoma. H. and E. × 202.
FIG. 17.—Cholangioma. Mucin in the lumen of bile ducts. PAS. × 50.



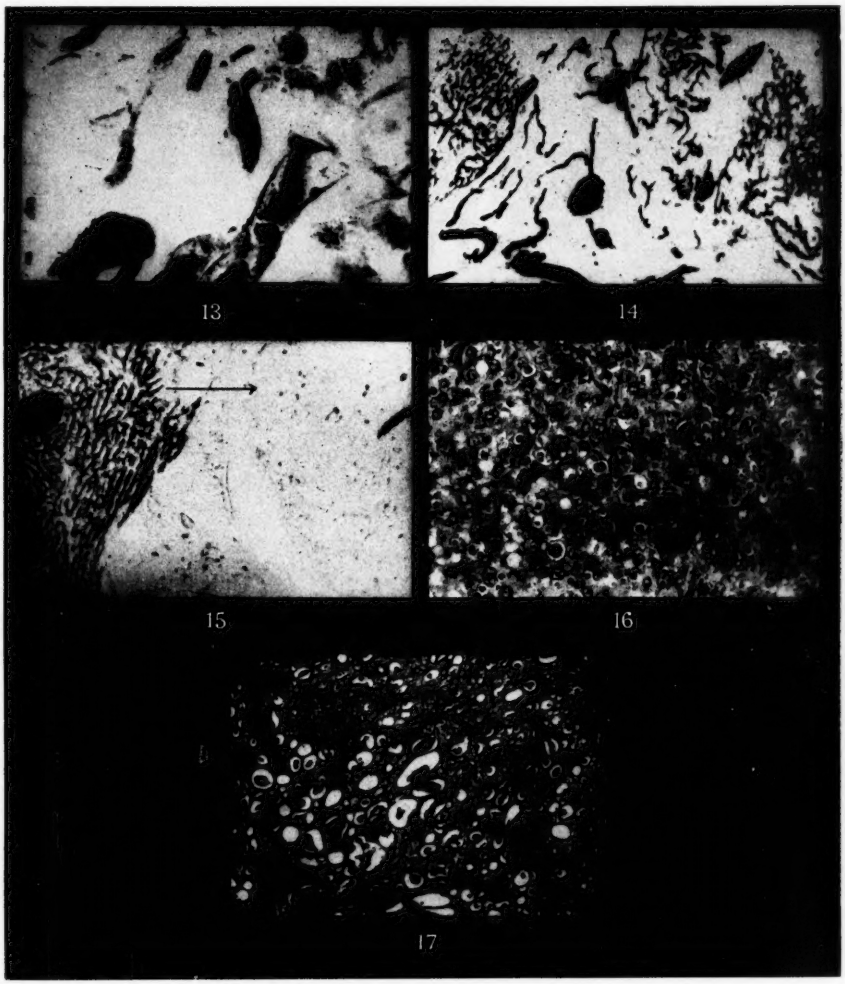
Krishna Murthy.



7 8

9 10

11 12



13

14

15

16

17

Vascular Pattern of the Primary Hepatoma

The histopathology of hepatomas induced by butter yellow has been well described by Opie (1944) and Orr (1940) and need not be repeated here. The proliferation of bile ducts starting around a portal triad is called by Opie " Cholangiofibrosis ". A wild proliferation of bile ducts with an increase in fibrous tissue stroma, anarchy of cells, mitotic figures and mucin are observed in a malignant cholangioma.

Vascular pattern at 30 *days*

The portal and hepatic veins were recognized and their relationship was well maintained. The sinusoids were uniformly filled with dye. There were a few sinusoids which had not retained the dye, but the endothelial cells were black, suggesting that the dye had passed through these sinusoids.

Histology.—There was no marked alteration of the parenchymal architecture. In the portal radicles were a few inflammatory cells, mainly of the mononuclear type. Sinusoids in the central hepatic zone were dilated and engorged with blood. Cytoplasm of cells in this zone were less eosinophilic and in some cases empty. Some nuclei were pyknotic. Glycogen distribution was uniform throughout the lobule (Fig. 3).

Vascular pattern at 60 *days*

The relationship between portal and hepatic veins was not maintained all through the section. The sinusoidal network, ramifying around the marginal distributing veins, was scantily filled with dye, while the sinusoids around the distributing vein not only retained the dye, but also were bloated (Fig. 4).

Histology.—Noteworthy changes in the liver were proliferation of fibrous tissue, progressive degenerative changes in cells of the periportal zone, a few acidophilic bodies and localization of glycogen in the periportal zone (Fig. 5).

Vascular pattern at 90 *days*

Relationship between big vessels was distinctly altered. Only terminal sinusoids, both in the periportal and pericentral areas, were scantily filled with the dye. There was no evidence of the dye having traversed some sinusoids (Fig. 6).

Histology.—Pseudolobules were formed and were being formed. A few nuclei were hyperplastic. There was little evidence of regeneration. Glycogen appeared to be restricted to the central zone.

Vascular pattern at 120 *days*

Large radicles of the conducting vein were filled with dye but only a minimal amount of the sinusoidal bed was so filled. The picture recalled to mind a tree with the trunk and major branches intact, but with the finer branches withered. At places there was an attempt at formation of vascular collaterals. The normal pattern was not discernible anywhere (Fig. 7).

Histology.—The parenchyma was dissected into pseudolobules. There was an increase of connective tissue in the portal triads, which was more pronounced in 2 animals. The number of mononuclear inflammatory cells had increased. Inter- and intralobular bile ducts showed signs of proliferation. Degeneration of

cells was more pronounced in the periportal zone and also in the neighbourhood of proliferating mesenchymal cells. A few acidophilic bodies were present. Nuclear changes were characterized by karyorrhexis and pyknosis. Some endothelial cells were swollen. Glycogen had disappeared from the degenerating cells. A few bile cysts were seen (Fig. 8).

Vascular pattern at 150 *days*

The relationship between portal and hepatic veins was distinctly altered. The sinusoidal filling was not uniform and many sinusoids were obliterated. New vascular shunts were present. Dye perfused through the portal vein could not be recognized either in the areas of cholangiofibrosis or in the cholangioma. However, ensnared in these areas were a few vessels and sinusoids which still retained the dye (Fig. 9).

Histology.—Typical postnecrotic scarring was observed in all animals. In 2 animals cholangiofibrosis was seen, and in other animals a benign cholangioma was present. An occasional mitotic figure was present in the cholangioma. The lumen of the bile ducts contained mucin, which was PAS positive (Fig. 10).

Vascular pattern at 180 *days*

The conducting and distributing veins were recognized. Sinusoids emerging from the marginal distributing veins still retained the dye. Certain areas were filled with dye and the sinusoidal network was far from distinct. New vascular shunts were being formed. The normal pattern was still maintained in a few areas.

Histology.—In all animals the liver presented either a picture of postnecrotic scarring or subacute hepatic necrosis. A few interlobular bile ducts were proliferating. A cholangioma was seen in one animal. Fat and bile cysts were present (Fig. 11).

Vascular pattern at 210 *days*

Normal pattern could not be recognized anywhere. Some of the conducting veins and their branches were tortuous and distorted. New vascular collaterals had formed. There were large cavernous vascular spaces in the tumour and they were not filled by dye perfused through the portal vein. Carmine was perfused in one animal through the hepatic vein in addition to the Indian ink injected into the portal vein. Carmine was recognized in the vascular spaces of the tumour (Fig. 12 and 13).

Histology.—A picture of postnecrotic scarring was seen in all animals. Both inter- and intralobular bile ducts had proliferated profusely. There was evidence of haemorrhage in some pseudolobules. Bile cysts and a few fat cysts were present. Tumour was of the mixed type. Malignant hepatoma, cholangioma and cystadenoma were recognized. Mucin was present in areas of bile duct cancer. Glycogen was conspicuous by its absence in cancer cells.

Vascular pattern at 240 *days*

In many areas, radicles of the big vessels were in close proximity, suggesting a collapse of parenchyma. There were tufts of sinusoids around some of the distributing veins. Big vascular shunts had formed and they ran along connective tissue septa. Vascular pattern in some pseudolobules appeared normal. Dye

perfused through the portal vein was not present in the tumour parenchyma and also in areas of necrosis (Fig. 14).

Histology.—Postnecrotic scarring was the lesion commonly encountered. There was evidence of haemorrhage and extensive fatty infiltration in some pseudolobules. In some places there was rapid atypical regeneration of liver cells. There was both inter- and intralobular bile duct proliferation. Mixed tumours were seen in all but 2 animals. Malignant hepatocellular carcinoma in which the cells are arranged in the form of sheaths was the predominant type.

Vascular pattern at 270, 300, 330 *and* 360 *days*

Vascular pattern in the animals of these 4 groups corresponded to the pattern seen in the previous group. The characteristic features were the collapse of parenchyma as indicated by a close proximity of the big vessels, new vascular shunts and absence of dye (perfused through the portal vein) in the vascular spaces of the tumour (Fig. 15).

Histology.—At 270 days : Postnecrotic scarring and atypical regeneration were observed. Bile ducts had proliferated profusely. There was haemorrhage in some pseudolobules and there were areas of necrosis as well. Cholangiofibrosis was seen in one animal. All the tumours were predominantly liver cell carcinomas. At 300 days : Postnecrotic scarring was seen in all animals. Tumours were of the mixed type. There was dense fibrous tissue stroma in the cholangioma. At 330 days : Subacute hepatic necrosis was observed in one animal. There were some hyperplastic cells, which could be mistaken for cancer cells. Tumour was of the mixed type. At 360 days : Liver cell carcinoma was present in 2 animals and a mixed tumour was present in the third. Despite the fact that the fourth animal was fed butter yellow for a year, there was a negligible amount of tumour tissue. All that could be seen was a disorderly proliferation of bile ducts in one area (Fig. 16 and 17).

DISCUSSION

The limitations of the technique are such that the observations must be interpreted with a certain amount of reservation.

This study reveals a progressive disorganization of the vascular pattern in the liver of rats fed with the carcinogen, butter yellow and that the tumour parenchyma is not supplied by portal blood.

In the livers of animals fed with the azo dye for the first 3 months, striking changes occurred in the vascular pattern as sinusoids in the periportal zone were not filled by the dye that was perfused through the portal vein. The periportal zone of cells in the rat are not adequately supplied by portal blood and are vulnerable to injury, this is further substantiated by the observation that these cells are the first to degenerate.

During the third to fifth months of feeding the carcinogen, connective tissue is being formed in the perilobular path. The sinusoids in the periportal zone are sparsely filled with the dye while those in the pericentral area are well filled. The proliferation of mesenchymal elements in the perilobular path results in a primary dissection of the lobule (Popper and Elias, 1955), and possibly marginal distributing veins and the sinusoids around them are entrapped in the strands of connective tissue.

The collapse of parenchyma was a feature frequently encountered from the fifth month onwards. The collapse was characterized by tortuosity and close approximation of big vessels. Parenchymal collapse may be brought about by any of the following factors : (i) Acute necrosis, (ii) regeneration, (iii) cysts and (iv) tumour.

(i) Acute necrosis : Diffuse liver cell damage as in massive necrosis is followed by parenchymal collapse.

(ii) One of the major factors which account for parenchymal collapse and distortion of the vascular bed is the regenerating nodules. Following necrosis of any type there is always an attempt at regeneration. Regeneration following liver injury is nodular and not lobular. Kelty, Baggenstoss and Butt (1950) convincingly demonstrated by wax models that the regenerating nodules exert pressure on the surviving parenchyma and are responsible for the vascular derangement.

(iii) and (iv) Bile cysts and neoplastic growths, both primary and secondary, exert pressure on the parenchyma culminating in a parenchymal collapse.

By about the fourth month, internal collateral circulation or " internal Eck fistulae " are established. Drainage of blood from the liver is impaired in cirrhosis and one of the possible incriminating factors in the pathogenesis of portal hypertension is a blockage of the outflow tract. The degenerating liver cells swell and block the sinusoidal bed, hence the blood that is brought into the liver tries to bypass the parenchyma ; the only way it can effectively do so is by the formation of intrahepatic portovenous anastomosis. Thus an internal collateral circulation is established. These vessels are not formed *de novo*. They are the few sinusoids which are ensnared in the connective tissue septa.

A lesion simulating subacute hepatic necrosis is observed during the fifth and sixth months in which there is a stasis of blood in sinusoids as the necrotic areas prevent free flow of blood in the liver. Formerly, stasis of blood except in cardiac fibrosis was ascribed to vasomotor activity, but, Maegraith and Andrews (1955) claim that " stasis in any visible sinusoid is a sign of liver injury and not vasomotor inactivity ".

In not one tumour-bearing animal in this series was the dye, injected into the portal vein, recognized in the tumour, although the dye could be seen in sinusoids adjacent to the tumour. In one animal, carmine was injected into the hepatic vein, in addition to the Indian ink perfused through the portal vein. The carmine thus injected was recognized in the vascular spaces of the tumour. In another animal Indian ink was injected into the hepatic vein ; the dye was present in the tumour.

Apparently the only source of blood supply to the tumour is from the hepatic artery, although attempts made to perfuse a dye through the hepatic artery were successful in only one animal. The present observations regarding the source of the blood supply to the liver tumour are in conformity with those made by Mann, Wakim and Baggenstoss (1953) and Breedis and Young (1954). Mann *et al.* (1953) demonstrated, by employing the corrosion-cast method, that the blood supply to the primary carcinoma of liver following haemochromatosis is predominantly arterial. Breedis and Young (1954) observed that liver tumours of various animals and the secondary tumours of liver in man, received their blood supply from the hepatic artery.

There does not appear to be any obstruction to the free flow of portal blood into the tumour. During the fifth and sixth months of feeding the carcinogen,

cholangiofibrosis is observed for the first time. This lesion is supposed to be a forerunner of cholangioma. The dye perfused through the portal vein was not recognized in this lesion and only the vessels and sinusoids that were entrapped, retained the dye.

It is probable that the factor determining the source of blood supply to the liver tumour is more physiological than mechanical. The precancerous lesion which is either a disorderly proliferation of bile ducts or atypical hyperplasia of liver cells, is not supplied with portal but with arterial blood. There is also convincing evidence that the regenerating nodules are supplied by arterial blood for some time.

Grindlay and Bollman (1952) and Child (1954) have carried out extensive studies on the regeneration of liver following partial hepatectomy. They have observed that the liver must be filled with venous blood for regeneration to occur. Child emphasises that " the volume of blood delivered to the liver is more important than its source ". Rappaport, MacDonald and Boroughy (1953) and Rappaport, Lotto and Lougheed (1954) also have worked on this problem of regeneration with the idea of inducing hepatic coma in the animals and to elucidate the pathogenesis of hepatic coma. They have stressed the importance of arterial blood for regeneration and they deprecate the tendency among surgeons to ligate the hepatic artery to control portal hypertension. They argue that by ligating the hepatic artery, the liver cells are not able to regenerate. They have arrived at the conclusion, after conducting a series of experiments, that the sequelae following ligation of hepatic artery may end in frank ischaemic necrosis or hepatic coma.

Observations made here viewed in the light of work done by others tend to favour the opinion that the hyperplastic liver cells, either normal or neoplastic depend on the hepatic artery for their blood supply.

SUMMARY

Experiments were carried out to study the vascular pattern of liver tumours in rats induced by the azo dye—butter yellow. Indian ink was perfused through the portal vein for the study of the vascular pattern. In the early stages, the periportal sinusoids were not filled by the dye. Cells in this zone were the first to be damaged. The dye perfused through the portal vein could not be recognized in the lesion, cholangiofibrosis, nor was it present in the vascular spaces of the tumour. It is concluded that the liver tumour depends on the hepatic artery for its blood supply and not on the portal vein.

I am deeply indebted to Dr. V. R. Khanolkar, Director of the Indian Cancer Research Centre for his invaluable suggestions and keen interest in this work.

I am also grateful to Dr. B. K. Aikat, Director of the Department of Pathology and Bacteriology, Institute of Post-graduate Medical Education and Research, Calcutta, for his constructive criticisms. I have pleasure in acknowledging the technical help of my colleague and friend, Mr. B. N. Vithal.

REFERENCES

ANDREWS, W. H. H., MAEGRAITH, B. G. AND WENYON, C. E. M.—(1949) *Ann. trop. Med. Parasit.*, **43**, 229.

BREEDIS, C. AND YOUNG, G.—(1954) *Amer. J. Path.*, **30**, 969.

CHILD, C. G.—(1954) 'The Hepatic Circulation and Portal Hypertension'. Philadelphia and London (W. B. Saunders).

DANIEL, P. M., PRICHARD, M. M. L. AND REYNELL, P. C.—(1952) *J. Path. Bact.*, **64**, 53.
ELIAS, H.—(1953) ' Functional Morphology of the Liver '. Chicago (G. D. Searle & Co.).
Idem AND POPPER, H.—(1955) *Arch. Path.*, **59**, 332.
GRINDLAY, J. H. AND BOLLMAN, J. L.—(1952) *Surg. Gynec. Obstet.*, **94**, 491.
HERRICK, F. C.—(1907) *J. exp. Med.*, **9**, 93.
KELTY, R. H., BAGGENSTOSS, A. H. AND BUTT, H. L.—(1950) *Gastroenterology*, **15**, 285.
KNISELY, M. H.—(1948) *Biol. Skr.*, **4** (7), 1.
MANN, J. D., WAKIM, K. G. AND BAGGENSTOSS, A. H.—(1953) *Gastroenterology*, **25**, 540.
MCINDOE, A. H.—(1928) *Arch. Path.*, **5**, 22.
MAEGRAITH, B. G. AND ANDREWS, W. H. H.—(1955) Quoted by Brauer in *J. nat. Cancer Inst.*, **15**, 1469.
OPIE, E. L.—(1944) *J. exp. Med.*, **80**, 231.
ORR, J. W.—(1940) *J. Path. Bact.*, **50**, 393.
POPPER, H. AND ELIAS, H.—(1955) *Amer. J. Path.*, **31**, 405.
RAPPAPORT, A. M., MACDONALD, M. H. AND BOROUGHY, Z. J.—(1953) *Surg. Gynec. Obstet.*, **97**, 748.
Idem, LOTTO, W. N. AND LOUGHEED, W. M.—(1954) *Ann. Surg.*, **140**, 695.
SENEVIRATNE, R. D.—(1949) *Quart. J. exp. Physiol.*, **35**, 77.
WAKIM, K. G. AND MANN, F. C.—(1942) *Anat. Rec.*, **82**, 233.

STUDIES ON THE MULTIPLICATION OF *MYCOBACTERIUM LEPRAEMURIUM* IN THE MOUSE CORNEA

J. M. ROBSON AND J. T. SMITH

From the Department of Pharmacology, Guy's Hospital Medical School, London

Received for publication August 9, 1958

IT has previously been shown that when *Mycobacterium lepraemurium* is injected into the cornea of mice, progressive lesions develop which spread systemically and kill the animals; this method has been used in the investigation of chemotherapeutic substances (Goulding, Robson and Rees, 1953; Naguib and Robson, 1956*a*). No detailed examination of the infection in the cornea was performed. In the present investigation, techniques elaborated for the examination of tuberculous infections of the cornea have been applied to infections produced by *Myco. lepraemurium*. In addition, a method of counting the number of organisms in the cornea was developed and this was used to follow the process of bacterial multiplication, side by side with the spread of the infection.

MATERIALS AND METHODS

White mice weighing about 20 g. at the beginning of the experiment were used. The suspension of *Myco. lepraemurium* used for inoculation was prepared, as described by Naguib and Robson (1956*b*) from mice which had previously been infected by the intravenous route. The lesions which developed in the cornea were investigated at various stages after inoculation by the following methods:

(1) Size of the macroscopic lesion as followed quantitatively by the method previously described by Rees and Robson (1950) and Naguib (1956).

(2) Phase contrast examination of the isolated cornea (Robson and Didcock, 1955).

(3) Staining of the whole cornea by a modified Ziehl Neelsen technique, as described by Robson and Sullivan (1957).

(4) A new method not previously described has been devised for the counting of the total number of mycobacteria in the cornea. This is carried out as follows: The mouse is killed and the whole animal immersed for a few seconds in 1 per cent CTAB. The remainder of the procedure is carried out using an aseptic technique. With the eye left *in situ*, the cornea is removed and placed in sterile saline. The lens and iris which remain adherent to the rim of sclera which is removed with the cornea are dissected off. The cornea is then washed in sterile saline and placed in 1 ml. of phosphate buffer (pH 7·4) containing 0·3 mg. of collagenase (Worthington Biochemical Corporation) and 500 units of hyaluronidase (Hyalase, Benger). This enzyme mixture is previously sterilised by filtration. The tube containing the cornea and enzyme mixture is incubated for 18 hr. at room temperature (about 20°) and then shaken for 30 min. on a microid flask shaker. By the end of this procedure the whole of the cornea is fully digested by the enzyme mixture, but a rim of scleral tissue usually remains undigested. The total number of organisms in the mixture is now counted by the method of Hobby, Hanks, Donikan and Backerman (1954) using Rees' modification (personal communication).

The accuracy of this counting method for the present work was tested as follows:—Two corneas containing organisms were digested as described above and the material pooled. Six aliquots were taken and counted independently. The following results were obtained, expressed as million organisms per ml.: 59·9, 55·1, 57·1, 62·7, 61·7 and 55·5. The mean is 58·7 and the difference between the highest and lowest figures is about 15 per cent of the

mean. None of the results obtained differ by more than 6·8 per cent from the mean. Hobby *et al.* (1957) performed a number of duplicate counts of populations of mycobacteria in mouse spleen and found that departures from the mean as great as plus-minus 20 per cent occurred rarely.

At various stages after inoculation, post mortem smears of the lung, liver, spleen and cervical lymph gland were made and examined carefully for acid fast bacilli.

In order to follow the rate of multiplication of the organisms it was necessary to know accurately what inoculum was put into the cornea. The volume of the inoculum, which was previously estimated to be about 0·01 ml. was determined more accurately by three independent methods, namely :

(1) Radio-iodinated human serum albumin was injected and the amount of radioactivity in the injected corneas determined. Bleb sizes were deliberately graded from small to large. The volume of the inoculum (13 eyes) varied from 0·0002 ml. to 0·001 ml. with an average of 0·0006 ml. There was some correlation between bleb size and the volume of the inoculum.

(2) Dialysed iron (colloidal iron oxide) was injected and quantitative analysis of the iron carried out by a colorimetric method. Here the blebs were of the size usually made for corneal inoculation. The volume of the inoculum (18 eyes) varied from 0·0003 to 0·0018, with an average of 0·001 ml.

(3) One eye in each animal was injected with distilled water (large blebs being deliberately made in all cases) and both eyes of each animal were immediately removed and weighed, the difference in weight between the two eyes being taken to represent the weight of the inoculum. The volume of the inoculum by this method (6 eyes) ranged from 0·0002 to 0·0035, with an average of 0·0012.

Thus the volume of the inoculum may be regarded as having a mean of 0·001 ml., *i.e.* about one-tenth of our previous estimate.

RESULTS

Three experiments were performed. In the first the pathological changes in the cornea and total number of organisms found at various stages were investigated. The results obtained raised certain problems which made it necessary to do 2 further experiments.

In the first and main experiment, 2 groups of mice were inoculated intracorneally with *Myco. lepraemurium*, the first with an undiluted suspension (containing $1{\cdot}3 \times 10^{10}$ organisms per ml.) and the second with a 1 in 10 dilution of this suspension. Observations were continued for 164 days in the first group and for 96 days in the second group.

Microscopic findings

Both in the corneas injected with the undiluted suspension and with the 1/10 dilution, the organisms were rapidly taken up by phagocytes, mostly macrophages, although polymorphonuclear leucocytes and corneal corpuscles also played a part. Six days after inoculation many macrophages were packed with bacteria and about 12 days later macrophages were breaking down and liberating bacteria. This breakdown went on as the experiment continued and some 4 months after inoculation there were many extracellular organisms in the cornea.

In the early stages the infection was localised in a small part of the cornea, *i.e.* the site of inoculation, but it gradually spread through the cornea towards the sclera which became involved at about 5 weeks.

Examination for evidence of systemic spread was started 2 weeks after inoculation, but none was detected until 5–6 weeks later, *i.e.* shortly after the stage when the sclera had become involved, when mycobacteria were found in the cervical lymph nodes, spleen and liver. The lungs, however, did not become involved until about 4 months after infection.

Macroscopic lesions and number of organisms

These results are shown in Fig. 1. The eyes were first examined macroscopically on the seventh day after inoculation when small lesions were present ; these gradually increased in size and were consistently larger with the heavier inoculum. The results of the bacterial counts (each point representing one eye) were surprising since there was an initial marked fall in the number of bacteria in the cornea to a level below about 200,000 which was the least number which could be counted at that time. (Subsequently by centrifuging the material and using a lower power objective, the lower limit of the method was brought down to about

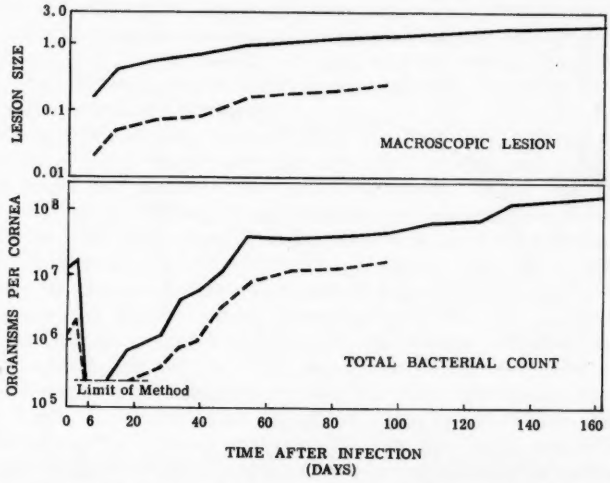


FIG. 1.—Showing number of bacteria in cornea and size of macroscopic lesion (mm.) at various stages after inoculation. Both sets of data plotted logarithmically against time.

———— Results with undiluted suspension.
– – – – – Results with 1/10 diluted suspension.

70,000.) The figures given for the day of inoculation were not actually counted, but were calculated from the count of the bacterial suspension used on the assumption that 0·001 ml. of this was injected into the cornea. From the sixth day after inoculation the total number of organisms in the cornea increased, rapidly until about the seventh week, and then more slowly. This applied both to the animals receiving the undiluted suspension and to those injected with the diluted suspension. The generation time for the part of the curve between 1 and 7 weeks was calculated to be just over 6 days, but was much longer (about 37 days) for the part of the curve after the seventh week.

Since the initial fall in the number of organisms was unexpected, the experiment was repeated. Again 2 groups of mice were injected intracorneally with an undiluted and a 1/10 diluted suspension. In this experiment eyes were removed immediately after inoculation to determine accurately the bacterial content at that time. The results are shown in Fig. 2, each point being the mean of 2 eyes. At the time of inoculation the bacterial contents of the 2 corneas

counted were respectively 75 million and 71 million for the undiluted suspension and 5 million and 7 million for the 1/10 dilution and since the suspension used contained 79 million organisms in 0·001 ml. this constitutes quite reasonable agreement. After inoculation there was an initial fall, though less striking than in the first experiment, followed by a rise. This experiment was continued for a shorter time, since its main purpose was to confirm the findings of an initial fall. Examination for systemic spread was made, starting 2 weeks after inoculation. At 23 days organisms were found in the spleen, liver and cervical lymph glands, but none were found in the lungs, even after 64 days when the experiment was terminated.

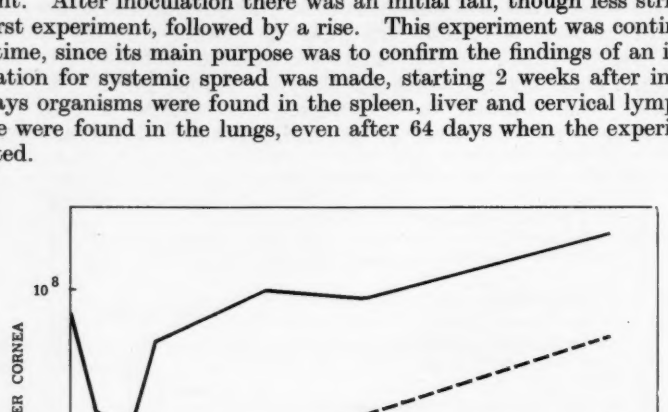


FIG. 2.—Showing number of bacteria in cornea, plotted logarithmically against time.
———— Results with undiluted suspension.
– – – – – Results with 1/10 diluted suspension.

Reason for initial fall in bacterial count

This might have been due to several reasons. It was possible that organisms became non-acid fast and thus were not counted in the smear. Phase contrast examination of the material, however, showed that no such organisms were present. This led to the conclusion that organisms escaped from the cornea. It seemed highly unlikely that this occurred through the sclera, since at this early stage the organisms are concentrated in a small area at the site of inoculation, with no evidence whatever of any spread towards the sclera. This left the possibility that the organisms escaped through the injection wound. We had considered this unlikely, since it is well known that corneal epithelium can rapidly regenerate ; however, we have found that when corneas are injected with saline, epithelial regeneration occurs more slowly than expected, and is not complete for 2–3 days. The next experiment was therefore planned to investigate whether organisms escaped through the injection wound.

A group of mice was injected intracorneally with an undiluted suspension of *Myco. lepraemurium* and the infection followed for the next fortnight by gross staining of the cornea, bacterial counts and intensive examination of various organs and tissues for acid fast organisms. In addition, the cornea was stained with fluorescein to follow the rate of healing of the epithelial wound.

Immediately after inoculation 2 eyes were removed and the cornea stained with the modified Ziehl Neelsen technique to show up acid fast organisms. The areas occupied by the main mass of organisms were respectively 3×1 and 3×2 mm. Counts made in another 2 corneas gave values of 76 million and 118 million organisms. The suspension used contained about 62 million organisms per 0·001 ml., a result which compared reasonably well with the corneal counts. Three days later 4 more mice were examined. The stained areas in 2 of them were respectively 2×1 mm. and 1×1 mm. and the counts in the other 2 were 40 million and 27 million, results which agree in showing a fall to about one-third in the bacterial content of the cornea.

Post mortem examination performed in the first few days after inoculation showed numerous mycobacteria in the conjunctival sac, many of them extracellular, but some in polymorphonuclear leucocytes. Acid fast bacteria were also found in the nose, trachea, lungs, cervical lymph glands and heart blood in small numbers. During the process of inoculation some of the bacterial suspension is inevitably split into the conjunctival sac, and it seemed possible that this might account for the extra-corneal organisms found at post-mortem. To check this, a suspension of *Myco. lepraemurium* was put into the conjunctival sac of some mice without an actual injection into the cornea. Examination of these animals 2 days later showed very few organisms in the conjunctival sac, all of them extracellular. Some bacteria, however, were also found in the lymph glands and liver. It would seem, therefore, that mycobacteria deposited into the conjunctival sac can spread systemically, but that such deposition does not account for the large number of organisms, some of them intracellular, found in the conjunctival sac after intracorneal inoculation, suggesting that most of these organisms originate from the inoculum deposited within the cornea. In agreement with this was the finding that the corneal epithelium showed no evidence of healing during the first few days after inoculation, as shown by the continued fluorescein staining of the injection wound. A week after inoculation, however, the epithelium had fully regenerated and at that time no more organisms were found in the conjunctival sac or in any other extra-ocular sites. In any animal in which epithelial regeneration had been completed, no mycobacteria were found in the conjunctival sac.

DISCUSSION

These experiments show that, following the intracorneal injection of a large inoculum of *Myco. lepraemurium*, many of the organisms escape through the injection wound during the first few days after inoculation ; many of them are extracellular, but some escape inside phagocytes. It is noteworthy that the organisms which so escape from the cornea become widely disseminated and can be found not only in the conjunctival sac, nose, trachea and lungs, but also in the cervical lymph glands and even in the heart blood.

After about a week the epithelium has regenerated and no further escape of organisms through the corneal surface occurs. From this time on there is a

rapid increase in the total number of organisms present in the cornea and this rate of increase is maintained for about 6 weeks. After this, however, the rate of increase slows down considerably and this slower rate was maintained until the end of the experiment. It is interesting that the development of the corneal lesions closely followed the increase in the corneal content of organisms. The curve of lesion size was also in 2 parts, a steep part corresponding to the period when organisms were rapidly increasing in the cornea and a flatter part when the increase was slower. It is interesting that in *Myco. lepraemurium* infections of the cornea, the curve of the lesion size correlates well with the total number of organisms present in the cornea.

The reason for the flattening of the curve of corneal bacterial content at about 6 weeks is at present unknown. It would be tempting to compare this with the findings in corneal tuberculosis, where there is a rapid increase in bacterial content followed by a gradual cessation of bacterial multiplication (Robson and Sullivan, 1957). There is good reason for believing that in tuberculosis, immunity processes are responsible for the cessation of bacterial multiplication (Robson, Sullivan and Didcock, 1957) and it is possible that the same applies to leprosy. It must, however, be remembered that it is at about 6 weeks, *i.e.* the time when the slope of the curve changes, that organisms reach the sclera in large numbers, and it may well be that the change in the curve is not due to a depression of the multiplication of organisms, but to a loss of them in fairly large numbers through the sclera. This question is being further investigated.

Previous investigations have shown that the development of macroscopic lesions in the cornea can be used to assess the value of chemotherapeutic substances effective against *Myco. lepraemurium*. The present results emphasise the validity of such a method, since there is good agreement between the multiplication of organisms within the cornea and the development of macroscopic lesions. Moreover, the use of the counting method will make it possible to study more accurately the effect of drugs on the multiplication of *Myco. lepraemurium* in mice and it is intended to perform such investigations.

SUMMARY

Mice were inoculated intracorneally with *Myco. lepraemurium* and the course of the disease followed with particular reference to the multiplication of organisms within the cornea. For this purpose the cornea was digested with an enzyme mixture and the acid fast organisms counted. It was found that for some days after inoculation there was a fall in the number of organisms in the cornea due to an escape through the infection wound. For the next 6 weeks the number of organisms rapidly rose, the generation time being about 6 days. Subsequently the increase in the number of organisms in the cornea was much slower. The curve of the number of organisms in the cornea (after the initial fall) agreed well with the curve representing the development of the macroscopic lesion in the cornea.

We are grateful to Mr. F. M. Sullivan for his valuable help, to the Colonial Medical Research Committee for a personal grant (to J. T. S.) and to the Medical Research Council for a grant (to J. M. R.) which defrayed in part the expenses of this work.

REFERENCES

GOULDING, R., ROBSON, J. M. AND REES, R. J. W.—(1953) *Lancet* i, 423.
HOBBY, G. L., HANKS, J. H., DONIKAN, M. A. AND BACKERMAN, T.—(1954) *Amer. Rev. Tuberc.*, **69**, 173.
NAGUIB, M.—(1956) Ph.D. Thesis, University of London.
Idem AND ROBSON, J. M.—(1956*a*) *Lancet*, i, 411.—(1956*b*) *J. Path. Bact.*, **72**, 657.
REES, R. J. W. AND ROBSON, J. M.—(1950) *Brit. J. Pharmacol.*, **5**, 77.
ROBSON, J. M. AND DIDCOCK, K. A. H.—(1955) *Brit. J. exp. Path.*, **36**, 560.
Idem AND SULLIVAN, F. M.—(1957) *Amer. Rev. Tuberc.*, **75**, 756.
Iidem AND DIDCOCK, K. A. H.—(1957) *Brit. J. exp. Path.*, **38**, 172.

THE EFFECT ON EXPERIMENTAL SILICOSIS OF HYPERSENSITIVITY INDUCED BY HORSE SERUM

D. E. B. POWELL AND J. GOUGH

From the Department of Pathology, Welsh National School of Medicine, Cardiff

Received for publication August 14, 1958

THE latest theory of silicosis is that it is a form of antigen antibody reaction. A new concept has become necessary because the solubility theory, which has been the most generally accepted during the past decade, is no longer tenable. It had been thought that silica compounds have a toxic action proportional to their degree of solubility, but so many exceptions have been found that this theory has now to be discarded in its simple form. There is evidence that silica can combine with body protein and act as an antigen (Antweiler and Hirsch, 1957). Moreover, silicotic nodules both in man and in experimental animals are not composed entirely of collagen as was formerly believed, but are mixtures of collagen and globulins, including γ-globulins (Ceppellini and Pernis, 1958). The reaction to silica in animals can be greatly modified by the administration of cortisone which is known to affect inflammatory reactions, including hypersensitivity ones. The object of the present experiments was to see whether the silicotic reaction could be modified by rendering animals hypersensitive, that is to say whether they would show the reverse effects from those due to the action of cortisone. Rabbits were given intratracheal injections of silica and then sensitized to horse serum. The resulting lesions were different from those of control animals and showed that there was a more vigorous response to the silica in the hypersensitive animals.

MATERIALS AND METHODS

Powdered quartz (" Snowit II " prepared from Belgian sand) of particle size 0·5-1μ was prepared by Dr. G. Nagelschmidt. This was suspended in normal saline in a concentration of 60 mg./ml. and was autoclaved before use.

Horse serum—Burroughs Wellcome No. 2 (No. E3225A).

Female rabbits of mixed breeds were used. The controls were matched as nearly as possible for breed and weight.

In the test series 8 rabbits were used, and 6 in the control.

Each test animal was given a single intratracheal injection of the silica suspension in a dosage of 1 ml./0·5 kg. body weight, under ether anaesthesia. Apart from one animal in which inadvertently the bulk of the injection was made into the paratracheal tissues, all tolerated this dosage with slight disturbance. Horse serum was given intravenously in a dosage of 10 ml./kg. body weight 7 days later. A state of hypersensitivity was maintained by further intravenous injections in the surviving animals of 1 ml. on the 22nd day ; the original dose on the 23rd ; 2 ml. on the 47th, 2 ml. on the 54th and half the original dose on the 61st and 96th days. During this regime 2 animals died from anaphylaxis. Precipitation titres were not followed throughout the period of the experiment, but all these rabbit sera gave positive precipitin reactions with horse serum.

The 6 control animals received the same dosage of intratracheal silica. All tolerated it well.

The test animals were killed at 27, 37, 57, 75, 96 and 110 days after the silica injection. The other 2 died at 30 and 61 days.

The control animals were killed at 28, 30, 37, 57, 75 and 96 days. All animals were killed by intravenous " Nembutal ".

Post-mortem examination was performed immediately after death. The lungs were inflated with formol-saline before cutting. Paraffin blocks were made and serial sections cut. The lung sections were stained by haematoxylin and eosin, Gomori's Reticulin stain and acid picro-Mallory using Masson's light green solution in place of Soluble Blue for the demonstration of connective tissue. Paraffin sections were also incinerated at 500° for 16 hr. and examined under direct and dark ground illumination.

Haematoxylin and eosin stained sections were prepared in each animal from heart, liver, kidneys, adrenals and spleen.

RESULTS

Both test and control animals showed well developed silicotic lesions in their lungs. The features particularly studied were the size and shape of the silicotic foci, the degree of mature collagen formation, reticulin fibre formation, and the distribution of the quartz particles.

The silicotic lesions in the hypersensitive animal were larger, more clearly demarcated and more localized than in the controls. Fig. 1 and 2 illustrate this at 27 days. The difference was most clearly seen in the animals killed at 27 and 30 days after the silica injection. Thereafter the foci in the controls also began to become more discrete, with some clearing of dust macrophages from the intervening lung parenchyma. However, even at 96 days there was still a difference in the degree of definition and aggregation of the foci (Fig. 3 and 4). The edges of the foci in the test animal were often formed by a collar of lymphocyte-type cells.

Acid picro-Mallory's stain showed that concentric layers of adult collagen were formed sooner and in greater quantity in the test animal (Fig. 5 and 6). Collagen developed more obviously in the controls after this time, but although considerable in amount at 96 days it still did not show the same regularity of concentric layering as in the hypersensitive animal.

Reticulin stains did not show any consistent quantitative difference, but again served to show the greater, more prominent, concentric arrangement in lesions in the test animals (Fig. 7 and 8).

These observed differences are subjective and therefore open to observer error. However, the difference seen in the distribution of dust after micro-incineration is particularly striking and supports the impression gained by histological study. Fig. 9 and 10 show the increased aggregation of dust in the hypersensitive animal at 27 days, with the control at 28 days. This is still clearly present at 75 days (Fig. 11 and 12).

No consistent differences were observed in the nature of the cellular reaction, or in the degree of secondary infection, which was slight and did not interfere in the reaction to quartz.

The heart, liver and kidneys in the test animals gave histological evidence of their state of hypersensitivity.

DISCUSSION

The effect of steroids on the host's response to the administration of silica has been the subject of considerable investigation. Magarey and Gough (1952*a*) demonstrated that cortisone inhibited the development of fibrosis in response to intraperitoneal silica in the mouse. The same authors (1952*b*) claimed that cortisone retarded the further development of concentric fibrosis around already established intraperitoneal silica granulomata in the rabbit. Harrison, King,

Dale and Sichel (1952), experimenting on rats given intratracheal silica injections, found that under the influence of cortisone dust cells remained more loosely scattered throughout the lung. This resulted in a retardation in the development of discrete silicotic nodules. They found little to suggest an inhibition of fibrosis. Marenghi and Rota (1954) exposed rats to clouds of finely dispersed quartz over prolonged periods. The effect of cortisone under these circumstances was to prevent or delay the transformation of reticulin into collagenous fibres, so that the nodules remained smaller in the cortisone group. King, Harrison and Attygale (1955) experimented on the established silicotic lesion in the lungs of rats. Cortisone given under these circumstances had little or no observable histological effect, but chemical tests proved to be more sensitive. These showed that the quantity of collagen found in the cortisone treated animals was less than in the control (Stacy and King, 1954). From these reports it is seen that the principal effects of cortisone on experimental silicosis is a diminution of collagen formation; a relative failure to aggregate the dust particles; and the formation of smaller and less well defined nodules. These features are precisely the opposite of those observed in the present experiment. This is in keeping with the known inhibitory effect of cortisone on the hypersensitivity state.

It is debatable whether the intravenous injection of foreign proteins *per se* gives rise to lung changes. A form of local Arthus reaction has been obtained by Opie (1924), Cannon, Walsh and Marshall (1941) and Fried (1933). Rich and Gregory (1943), Hawn and Janeway (1947), Ehrich, Seifter and Forman (1949) describe lung changes in the rabbit after intravenous injection of foreign proteins. However, Germuth (1953), Wissler, Smull and Lesh (1949) could not demonstrate lung changes after single or repeated intravenous injections of foreign protein. It may be concluded that if there are lung changes in the state of experimental serum sickness they are acute in nature and transient. No lung lesions were found in the present experiments solely attributable to the intravenous horse serum.

The mechanism of the modified response to silica found in the hypersensitive animal is uncertain but is probably due to increased activity of the macrophages. Dust is normally taken up by macrophages which migrate to the region of the

EXPLANATION OF PLATES

FIG. 1.—Lung in control animal killed 28 days after silica injection. H. and E. × 45.

FIG. 2.—Lung in hypersensitive animal killed 27 days after silica injection showing the larger, more clearly demarcated lesions. H. and E. × 45.

FIG. 3.—Control animal at 96 days. H. and E. × 88.

FIG. 4.—Test animal showing persistence of the better definition of lesions at 96 days. The collar of lymphocyte-type cells can also be seen. H. and E. × 88.

FIG. 5.—Poorly developed adult collagen tissue in control at 28 days. Acid picro-Mallory. × 88.

FIG. 6.—Well developed adult collagen tissue in hypersensitive animal at 27 days. Acid picro-Mallory. × 88.

FIG. 7.—Reticulin stain in control at 96 days. Gomori's Reticulin. × 88.

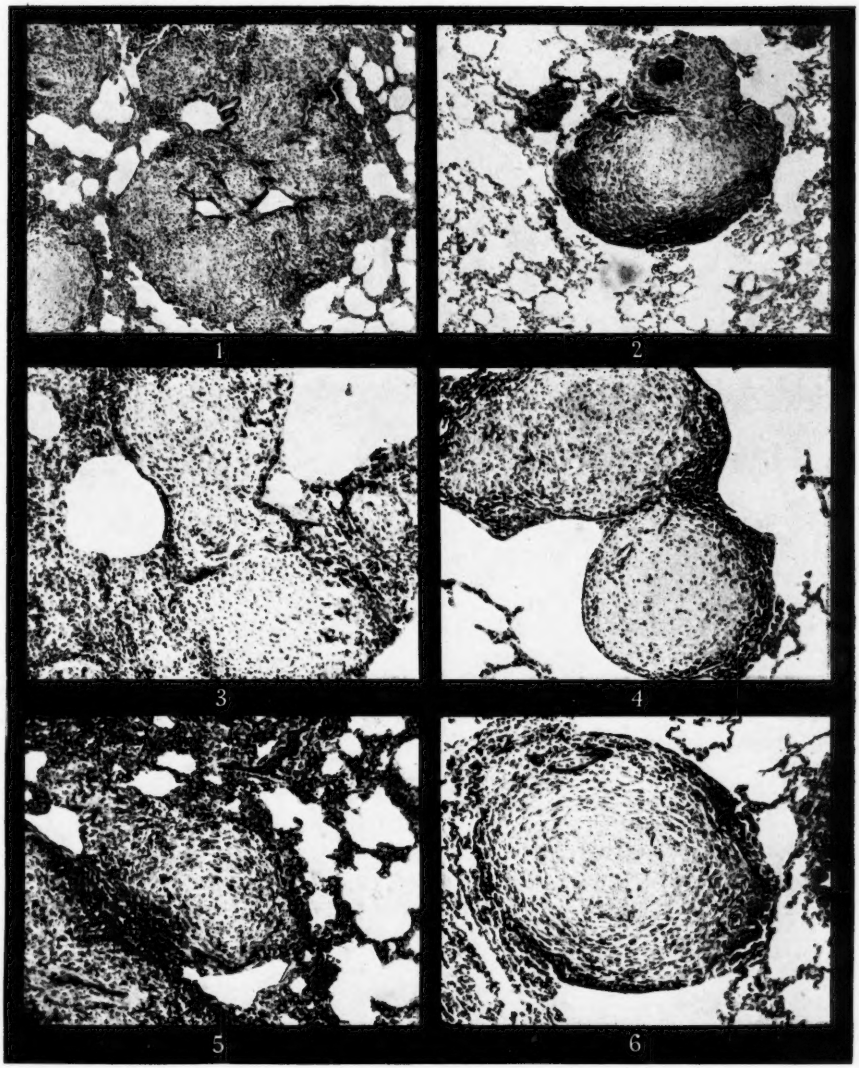
FIG. 8.—More prominent concentric arrangement of reticulin in the hypersensitive animal at 96 days. Gomori's Reticulin. × 88.

FIG. 9.—Distribution of dust in control at 28 days—after incineration. × 88.

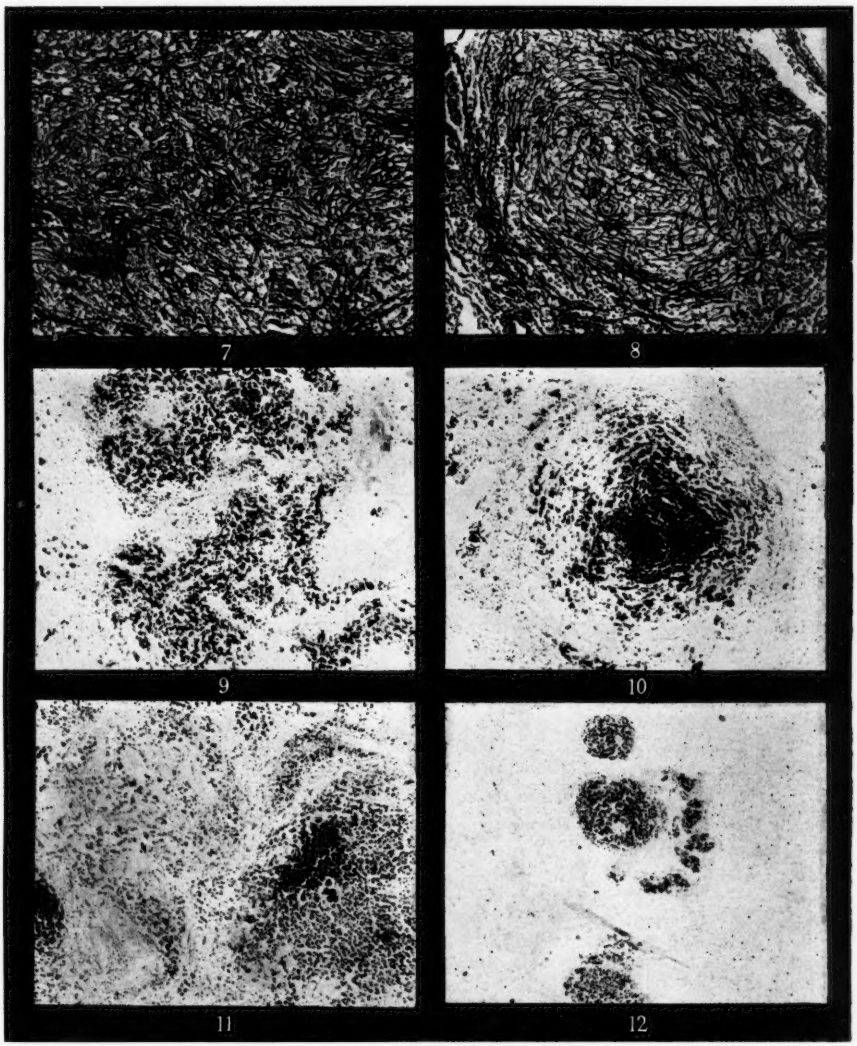
FIG. 10.—Distribution of dust in hypersensitive at 27 days—after incineration. The dust is more compact. × 88.

FIG. 11.—Distribution of dust in control at 75 days—after incineration. × 45.

FIG. 12.—Distribution of dust in hypersensitive after incineration. The dust still shows better aggregation at 75 days. × 45.



Powell and Gough.



7 8

9 10

11 12

respiratory bronchioles and accumulate there. This process of migration is retarded by cortisone but seems speeded up by hypersensitivity—this can be judged on the rate of clearing of the lung in between the foci of fibrosis. The extent and nature of the modification must necessarily be variable—as variable as is the response of the individual experimental animals to the intravenous injection of foreign proteins. The timing of silica and serum injections are also probably important variables. The influence on the silicotic lesion of steroids in the one direction and non-related antigen-antibody reactions in the other, may have a bearing on the explanation of the modification of clinical pneumoconiosis found, for example in association with rheumatoid arthritis (Caplan, 1953). They also afford a possible explanation of the variable reaction to silica in different individuals working under the same conditions.

SUMMARY

Experiments on rabbits showed that hypersensitivity produced by horse serum modified the reaction to quartz injected into the lungs.

The lesions were larger and more clearly demarcated. More collagen was found and the concentric arrangement was much more distinct.

Incinerated sections showed that the dust was accumulated into more compact foci.

These effects are the reverse of those due to cortisone.

We wish to thank Dr. G. Nagelschmidt for the silica preparation used in these experiments. The photographs are by Mr. J. P. Napper.

REFERENCES

Antweiler Von H. and Hirsch, E.—(1957) *Z. ImmunForsch.*, **114**, 378.
Cannon, P. R., Walsh, E. T., Marshall, C. E.—(1941) *Amer. J. Path.*, **17**, 777.
Caplan, A.—(1953) *Thorax*, **8**, 293.
Ceppellini, R. and Pernis, B.—(1958) *Nature*, **181**, 55.
Ehrich, W. E., Seifter, J. and Forman, C.—(1949) *J. exp. Med.*, **89**, 23.
Fried, B. M.—(1933) *Ibid.*, **57**, 111.
Germuth, F. R.—(1953) *Ibid.*, **97**, 257.
Harrison, C. V., King, E. J., Dale, J. C. and Sichel, R.—(1952) *Brit. J. industr. Med.*, **9**, 165.
Hawn, C. van Z. and Janeway, C. A.—(1947) *J. exp. Med.*, **85**, 571.
King, E. J., Harrison, C. V. and Attygale, D.—(1955) *Brit. J. industr. Med.*, **12**, 228.
Magarey, F. R. and Gough, J.—(1952*a*) *Brit. J. exp. Path.*, **33**, 510.—(1952*b*) *Ibid.*, **33**, 76.
Marenghi, B. and Rota, L.—(1954) *Arch. industr. Hyg.*, **9**, 315.
Opie, E. L.—(1924) *J. Immunol.*, **9**, 231.
Rich, A. R. and Gregory, J. E.—(1943) *Johns Hopk. Hosp. Bull.*, **73**, 239.
Stacy, B. D. and King, E. J.—(1954) *Brit. J. industr. Med.*, **11**, 192.
Wissler, R. W., Smull, K. and Lesh, J. B.—(1949) *J. exp. Med.*, **90**, 577.

THE NATURE OF THE GROUND SUBSTANCE CHANGES IN EXPERIMENTAL LATHYRISM AND THEIR EFFECT ON ATHEROGENESIS IN CHOLESTEROL FED RABBITS

C. J. SCHWARTZ*

From the Department of Human Physiology and Pharmacology, University of Adelaide, Australia†

Received for publication August 14, 1958

THE possible role of arterial wall mucopolysaccharide in atherogenesis was first suggested by Faber (1949) who concluded that cholesterol deposition occurs at those sites in which ethanol resistant metachromasia is present. Recently it has been demonstrated that the early atherosclerotic lesions of humans are associated with focal increases in metachromatically staining mucopolysaccharide (Taylor, 1953 ; Moon and Rinehart, 1952). Similar histochemical findings have since been noted in the spontaneous atheromata of rats (Schwartz and Johnson, unpublished) and in the lesions of cholesterol fed rabbits (Schwartz, Peters and Day, 1958). In the latter it was observed that an elevation of the serum hexosamine levels occurs *pari passu* with atherogenesis.

Such findings have suggested the possible role of ground substance mucopolysaccharide depolymerization in atherogenesis in the rabbit. That such a process may also be implicated in human atherosclerosis is supported by the observations of elevated serum mucoprotein and hexosamine levels in this disorder (Antonini and Salvini, 1957 ; Schwartz and Gilmore, 1958), while a low mucoprotein level has been noted in an ethnic group, the Australian aborigine, with a low atheroma incidence (Schwartz and Casley-Smith, 1958).

A constant feature of the aortic lesions of lathyrism in the rat is the presence of metachromatically staining mucopolysaccharide ground substance (Churchill, Gelfant, Lalich and Angevine, 1955 ; Bean and Ponseti, 1955 ; Bachhuber and Lalich, 1955 ; Pyörälä, Punsar, Seppälä and Karlsson, 1958 ; Menzies and Mills, 1957). This paper reports an evaluation of the role of the ground substance changes of lathyrism on experimental atherosclerosis in cholesterol fed rabbits, together with a study of the histopathology and biochemistry of lathyrism in this species.

MATERIALS AND METHODS

Twenty-four male chinchilla rabbits of approximately 6 months of age with weights varying from 1·2 to 1·8 kg. were paired according to weight and then randomly divided into 3 groups of 10, 10, and 4 animals respectively. Both groups of 10 were given a daily diet consisting of 75 g. mash, 6 ml. peanut oil and 1 g. cholesterol for a total period of 56 days, while the third group of 4 animals received only the mash diet without a cholesterol-oil supplement.

* Medical Research Fellow, National Health and Medical Research Council of Australia.
† Present address: Department of Medicine, University of Oxford.

The test group of 10 animals received a daily subcutaneous injection of 50 mg. of β-aminopropionitrile (BAPN), supplied through the courtesy of Dr. D. V. Frost of Abbott Laboratories, North Chicago, Illinois, U.S.A., in 1 ml. of 0·85 per cent saline for a total period of 56 days, while the control group of 10 received an equivalent injection of saline alone. The third group of 4 animals received no treatment.

Venous blood samples were collected from the marginal ear veins at the commencement of the experiment and after 14, 28 and 56 days of treatment. Sera were obtained by centrifugation at 3000 r.p.m. for 10 min.

Systolic blood pressure was measured at the commencement of the experiment and at weekly intervals using a modification of the ear capsule of Grant and Rothschild (1934).

Serum cholesterol and phospholipid were determined according to the methods of Zlatkis, Zak and Boyle (1953) and Brown (1954) using a 3 : 1 ethanol ether extract of the serum. Aliquots of the ethanol ether extract were evaporated to dryness in a water bath at 70° and the residue dissolved in 6 ml. of glacial acetic acid over 2 hr. Cholesterol was then determined according to the method as described in a previous paper (Day, Wilkinson and Schwartz, 1956).

Serum hexosamine was determined using a modification of the method of West and Clarke (1938) as previously described (Schwartz and Gilmore, 1958).

Serum mucoprotein was estimated using a modification of the method of Winzler, Devor, Mehl and Smyth (1948) and the protein content was determined by the method of Lowry, Roseborough, Farr and Randall (1951).

Aortic atheroma and lipoidosis.—Macroscopic aortic atheroma was assessed on the fresh unstained specimens according to the criteria of Day and Wilkinson (1956). The number of macroscopic aortic lesions was also recorded and the extent and severity of atheromata assessed microscopically.

Aortic intimal lipoidosis was graded in 3 categories on an arbitrary basis as follows. *Mild* (+) 1–3 small foam cell plaques visible throughout the length of the aorta. *Moderate* (++) 4–6 small foam cell plaques, or fewer and larger lesions. *Severe* (+++) 6 or more large foam cell plaques, or large confluent areas of lipoidosis.

Microscopic aortic atheroma grade was assessed on the following criteria. *Mild* (+) Hyaline fibrocellular intimal thickening and intimal foam cells. *Moderate* (++) Intimal foam cells, extensive fibrocellular proliferation, and a derangement of the normal medial architecture. *Severe* (+++) Extensive foam cell formation and fibrocellular proliferation, a derangement of medial architecture and calcification of lesions.

Histopathology.—After the animals were killed by intravenous pentone, the whole length of the aorta from the root to the bifurcation was removed, aortic atheroma graded, and the aorta rapidly fixed in 10 per cent neutral formalin. Following fixation, the aortae were stripped of adventitial debris and then coiled longitudinally. Multiple 6 μ longitudinal paraffin sections were prepared and stained with haematoxylin and eosin (H. and E.), toluidine blue (TB) for metachromasia, and periodic acid Schiff (PAS).

TABLE I.— *The Effect of BAPN on the Macroscopic Aortic Atheroma Grade and the Number of Discrete Lesions in Cholesterol Fed Rabbits*

Normal feeding			Cholesterol feeding			Cholesterol. feeding + BAPN		
Rabbit No.	Ath. grade	Number of lesions	Rabbit No.	Ath. grade	Number of lesions	Rabbit No.	Ath. grade	Number of lesions
21	0	0	11	I–II	15	1	II–III	25
22	0–I	5	12	II	12	2	III	25
23	0–I	2	13	II	20	3	II–III	20
24	0–I	2	14	II–III	15	4	II–III	20
			15	I–II	10	5	III	35
			16	I	7	6	I–II	7
			17	I–II	15	7	IV	35
			18	0–I	2	8	II	18
			19	II	12	9	II	12
			20	I–II	10	10	I–II	20

RESULTS

Aortic atheroma

Table I depicts the distribution of macroscopic aortic atheroma grades and the number of discrete atherosclerotic lesions in the 3 groups of animals. The highest recorded grades of atheroma were present in the group receiving BAPN, and 3 of the 4 non-cholesterol-fed animals had minimal spontaneous lesions. The number of discrete aortic lesions was greater in the BAPN test group. No difference in the site localization of lesions in the two groups was noted, the majority of the lesions in each case being present in the aortic arch and thoracic segment with a few only in the abdominal aorta, particularly at the point where the aorta traverses the diaphragm. Gross calcification of lesions was noted in 4 of the BAPN test groups but not in any of the control aortae.

Histopathology

The lathyrogenic effect of BAPN on the aortae of the test animals was considerable. Lesions characterized by an abnormal increased separation of the elastic fibres of the inner two-thirds of the media in the aortic arch and thoracic aorta were noted, with a disorientation of their normal alignment (Fig. 1). In the early lesions there was no evidence of an elastolysis but in the more advanced lesions this change was apparent (Fig. 2). Mononuclear inflammatory cell infiltration in and around these lesions was seen. These lathyrus lesions contained a considerable excess of metachromatic material which was in some instance markedly PAS positive. This material was observed between the widely separated elastic fibres of the subintima and media, and was particularly intense adjacent to the elastic fibres and the interlaminar connecting fibrils. With the H. and E. stain it appeared as a pale eosinophilic hyaline material.

Both the microscopic severity of aortic atherosclerosis and intimal foam-cell lipoidosis are summarized in Table II. It can be seen that both microscopic

TABLE II.— *The Effect of Cholesterol Feeding and BAPN on the Microscopic Severity of Atherosclerosis and Intimal Lipoidosis*

Normal feeding			Cholesterol feeding			Cholesterol feeding + BAPN		
Rabbit No.	Atheroma	Lipoidosis	Rabbit No.	Atheroma	Lipoidosis	Rabbit No.	Atheroma	Lipoidosis
21	0	0	11	+	+	1	++	+++
22	0	0	12	++	+	2	+++	++
23	+	0	13	+	++	3	++	+++
24	+	0	14	++	+++	4	++	+++
			15	+	0	5	+++	+
			16	++	+	6	++	+
			17	+	+	7	+++	+++
			18	+	0	8	++	++
			19	+	+	9	+++	+++
			20	++	+	10	++	+

atheroma severity and the extent of intimal lipoidosis (Fig. 3, 4) were increased by BAPN treatment. In addition, calcification of lesions was noted in 4 of the rabbits receiving BAPN (Fig. 5). This calcium deposition was found closely

applied to the medial elastic fibres. Not only was there an overall increase in intimal lipoidosis, but foam cell plaques were in many instances localized at the sites of metachromasia associated with the lathyrus lesions (Fig. 2, 6).

Within the aortae of the normal-fed rabbits the normal distribution of PAS positive and metachromatic material was noted to be more copious in the arch and thoracic end of the aorta than in the abdominal segment. The spontaneous atheromata in these animals contained definite focal increases in metachromatic staining, and PAS positive material. Similar histochemical findings were also

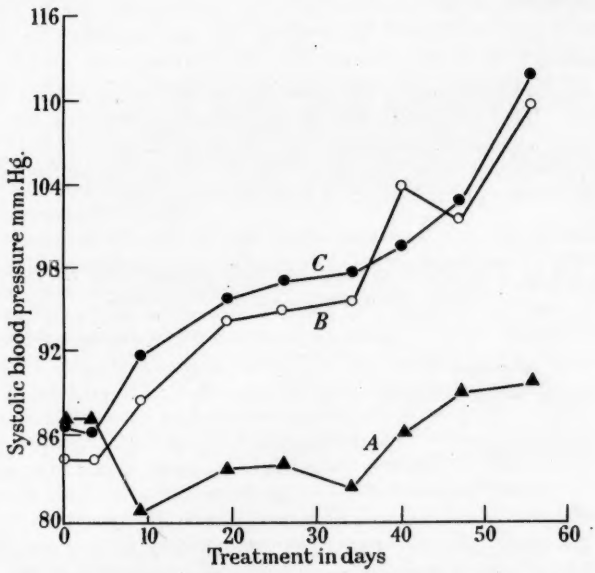


FIG. 7.—The effect of Cholesterol feeding and BAPN treatment on the systolic blood pressure of the rabbit.

A—Normal feeding.
B—Cholesterol feeding.
C—Cholesterol feeding plus BAPN.

obtained in many of the atherosclerotic lesions of the cholesterol-fed rabbits, where it was observed that with increasing lipid deposition and fibrosis the focal increase in metachromasia was less apparent. It was further observed that foam cells appear to form *in situ* from the fibroblasts of the thickened intima by a process of vacuolation.

Systolic blood pressure

Fig. 7 shows the effect of both cholesterol feeding and BAPN on the systolic blood pressure of the rabbit. While the blood pressure of the normal-fed rabbit was relatively constant over the 56 days of treatment, a rise of similar degree occurred in both groups on cholesterol diets, excluding any effect of BAPN itself.

Skeletal deformity and evidence of osteolathyrism

Six of the animals of the BAPN group developed skeletal abnormalities including kyphoscoliosis of the thoracic vertebrae, and dislocation of the hip joint. Four of the animals developed a hind limb paralysis. No dissecting aortic aneurysms were noted.

Serum lipids

BAPN treatment caused no significant changes in either total serum cholesterol or phospholipid levels.

Serum hexosamine

Table III shows that a rise in the mean serum hexosamine level of the control group occurred. This elevation due to hypercholesterolaemia at 56 days was highly significant when compared with the initial levels ($p = 0{\cdot}001$). Elevations due to BAPN treatment over the control levels at 14, 28, and 56 days were all statistically significant ($p = 0{\cdot}01$, $p = 0{\cdot}02$, and $p = 0{\cdot}001$).

TABLE III.— *The Effects of Hypercholesterolaemia and BAPN on the Serum Hexosamine Levels in Rabbits*

Treatment	Duration of treatment in days			
	0	14	28	56
Cholesterol feeding + BAPN	79·7 ±2·14	91·1 ±2·54	90·7 ±2·58	115·9 ±2·48
Cholesterol feeding	78·0 ±1·34	80·6 ±2·42	81·9 ±3·12	96·9 ±1·68

Serum mucoprotein

The data are summarized in Table IV. No significant elevation of the serum mucoprotein levels with hypercholesterolaemia was noted, but the rises due to BAPN at 14 and 28 days were highly significant ($p = 0{\cdot}01$, and $p = 0{\cdot}001$).

TABLE IV.— *The Effects of BAPN and Hypercholesterolaemia on the Serum Mucoprotein Levels in Rabbits*

Treatment	Duration of treatment in days			
	0	14	28	56
Cholesterol feeding + BAPN	111·5 ±6·58	129·5 ±5·73	143·9 ±6·50	126·7 ±8·44
Cholesterol feeding	107·7 ±6·96	96·4 ±9·37	115·5 ±11·05	113·0 ±6·80

DISCUSSION

In this paper both skeletal and aortic evidence of lathyrism induced by BAPN were noted, indicating that the rabbit can be successfully used in the study of this disease. The aortic lesions observed did not differ from those found in

rats by other workers. These lesions likewise demonstrated a marked focal increase in metachromatic staining, and in some instances definite increases in PAS positive material. In the later lesions there was definite evidence of an elastolysis, together with an extensive fibroblastic reparative process (Fig. 2).

The nature of the basic defect in lathyrism is ill understood. Mucopolysaccharide ground substance has been implicated by some workers. Pyörälä, Punsar and Seppälä (1958) have suggested that lathyrism reflects a disturbance in mucopolysaccharide metabolism, while Bean and Ponseti (1955) concluded that the lesions in lathyritic rats are due to a destruction of the chondroitin sulphuric acid of the connective tissue ground substance. That such a possibility is likely is suggested by the biochemical findings of significant elevations of both the serum mucoprotein and hexosamine levels in the lathyrus group. Elevations of both these fractions can probably be regarded as an index of the state of depolymerization of ground substance mucopolysaccharides *in vivo* (Schwartz and Gilmore, 1958). These results suggest that the metachromasia of the lathyrus lesion may well represent an abnormal depolymerization of the ground substance which itself is reflected in elevations of both the serum mucoprotein and hexosamine levels.

Both the severity of microscopic atheroma and the extent and severity of intimal foam-cell lipoidosis were enhanced by the induction of lathyrism. That these changes were not influenced by elevated cholesterol values nor an elevated blood pressure have been excluded in this work, since it has been shown that an elevated blood pressure facilitates atherogenesis in the cholesterol fed rabbit (Bronte-Stewart and Heptinstall, 1954). Not only was the amount of aortic lipoidosis increased by BAPN treatment, but the focal deposition of lipid at the site of subjacent lathyrus lesions was apparent (Fig. 2, 6). These findings are considered to support the view of Faber (1948) that ethanol resistant metachromatic mucopolysaccharide aids in the tissue deposition and fixation of lipids. That calcium deposition in tissues may occur in a similar manner is also possible. These findings contrast with the results of Meyer and Vos̈ (1956) who showed that lathyrism did not influence atherogenesis in the rat. The present results directly implicate a tissue factor in atherogenesis, and it is suggested that this tissue factor, whatever its cause, is characterized by an increase in toluidine blue metachromasia. In the case of lathyrism this probably reflects a destruction or depolymerization of arterial wall mucopolysaccharide. Similar increases in metachromasia have previously been noted in the atheromata of cholesterol fed rabbits by Schwartz, Peters and Day (1958) and in the spontaneous atheromata of rats (Schwartz and Johnson, unpublished). Similar changes in the ground substance of human atherosclerosis have been demonstrated by Taylor (1953) and Moon and Rinehart (1952). These histochemical demonstrations of focal metachromatic increases are supported by the recent findings of elevated serum mucoprotein and hexosamine levels in human atherosclerosis (Antonini and Salvini, 1957 ; Schwartz and Gilmore, 1958). On the basis of these combined findings it is suggested that a mucopolysaccharide anomaly occurs in atherogenesis, and that it probably reflects an increased depolymerization of arterial wall ground substance glycoproteins.

Evidence which further indirectly supports the hypothesis that abnormal mucopolysaccharide metabolism plays an atherogenic role are the findings of elevated serum or tissue mucoprotein and hexosamine levels in those situations

which facilitate the development of atherosclerosis, namely: myxoedema (Mancini, Garberi and de la Balze, 1951), hypothyroidism in rats (Boas and Foley, 1955), DOCA-saline treated rabbits (Schwartz, unpublished), scorbutic animals (Robb-Smith, 1954) and diabetes mellitus (Jacobs, 1949; Keiding, 1954; Nielsen and Poulsen, 1954; Schwartz and Gilmore, 1959).

Apart from the possible effect of metachromatic material upon lipid and calcium deposition in tissues, it is possible that this metachromatic mucopolysaccharide may also aid in the atherogenic process as a precursor of collagen fibres. Such a role of metachromatic material has been suggested by Taylor and Saunders (1957). Of related interest is the observation of Watson and Pearce (1950) that in pretibial myxoedema progressive fibrosis is associated with a diminution of tissue mucopolysaccharides.

SUMMARY

Skeletal and aortic lesions typical of lathyrism have been produced in cholesterol fed rabbits by the subcutaneous administration of β-aminopropionitrile fumarate.

The aortic lathyrus lesions were characterized by an increased separation of the medial elastic fibres, elastolysis, and an increase in metachromatically staining material which was in some cases PAS positive.

The induction of lathyrism was accompanied by significant increases in the mean levels of serum hexosamine and mucoprotein.

These elevations were interpreted jointly with the histochemical findings as reflecting an increased destruction or depolymerization of the aortic ground substance in lathyrism.

Lipoidosis as foam cells was facilitated in the lathyritic aortae, and foam cell formation was conspicuously localized at the sites of subjacent lathyrus lesions. Calcification was also enhanced by this treatment.

The findings of this work are discussed in terms of the possible role of metachromatic mucopolysaccharide as the tissue factor controlling lipid deposition, fibrogenesis and calcification in atherogenesis.

EXPLANATION OF PLATE.

FIG. 1.—Rabbit aorta (H. and E.). 156 × (BAPN Group). A characteristic lathyrus lesion can be seen with an increased separation of the medial fibres, excessive fibrosis, and distortion of the medial architecture.

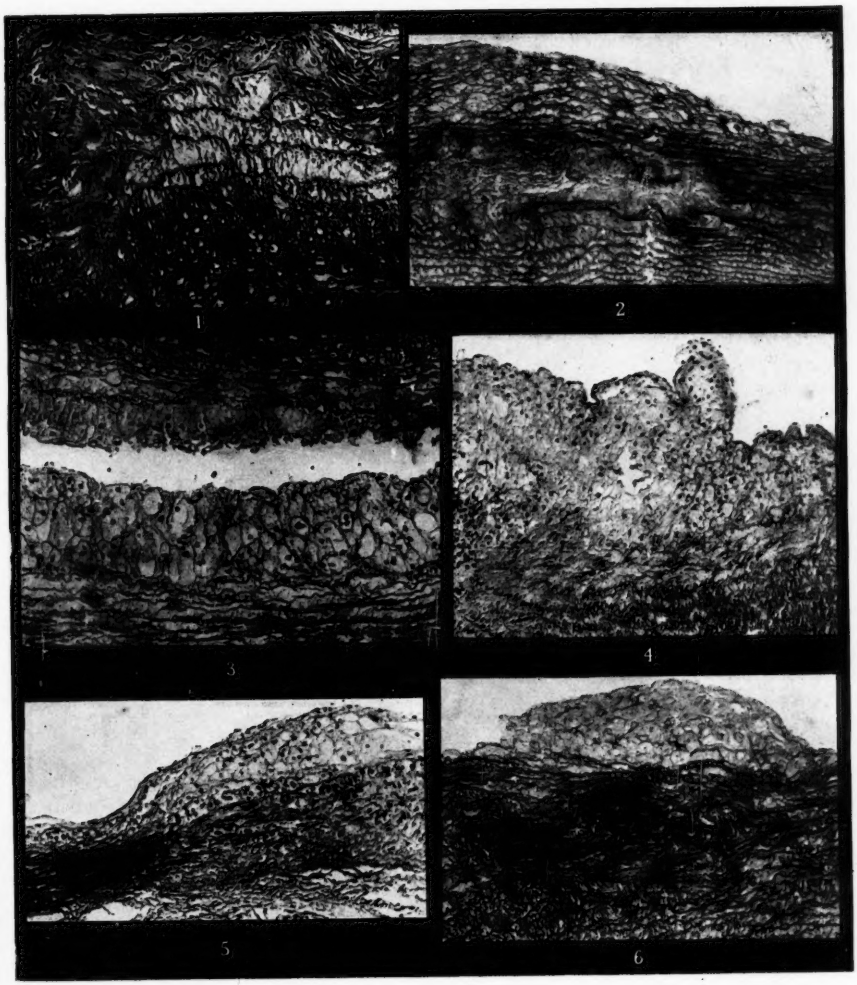
FIG. 2.—Rabbit aorta (PAS). 156 × (BAPN Group). A lathyrus lesion of the aortic media is seen, with a localized foam cell plaque upon it. The extensive separation and fragmentation of the elastic fibres is apparent. Closely applied to some of the fragmented elastic fibres, is an abnormal increase in PAS positive material.

FIG. 3.—Rabbit aorta (H. and E.). 260 × (BAPN Group). A segment of the diffuse intimal foam cell lipoidosis is seen. Subjacent is an increased separation of the elastic fibres.

FIG. 4.—Rabbit aorta (H. and E.). 156 × (BAPN Group). A segment of the extensive foam cell formation is seen, superimposed upon an early lathyrus lesion at the site of aortic metachromasia.

FIG. 5.—Rabbit aorta (H. and E.). 156 × (BAPN Group). An area of calcification and atheromatous change is seen. The calcium deposition is closely applied to the intensely metachromatic elastic fibres, and the aortic wall is considerably thinned at the site of calcification.

FIG. 6.—Rabbit aorta (PAS). 208 × (BAPN Group). A small foam cell plaque can be seen localized at the site of a subjacent lathyrus lesion. Disorganization of the elastic fibres is seen.



Schwartz.

The author is indebted to Miss M. Redway and Mr. J. A. Peters for technical assistance; to Miss E. Wadlow of the Commonwealth Antioxidant Research Project for the excellence of the sections which she prepared; to Mr. J. R. Casley-Smith for the statistical analysis of results, to Miss M. Dopson and Miss S. Fitzgerald, and finally to Dr. Douglas V. Frost of Abbott Laboratories, North Chicago, Illinois, U.S.A. for the generous gift of β-aminopropionitrile fumarate.

REFERENCES

ANTONINI, F. M. AND SALVINI, L.—(1957) *Bull. schweiz. Akad. med. Wiss.*, **13**, 209.
BACHHUBER, T. E. AND LALICH, J. J.—(1955) *Arch. Path.*, **59**, 247.
BEAN, W. B. AND PONSETI, I. V.—(1955) *Circulation*, **12**, 185.
BOAS, N. F. AND FOLEY, J. B.—(1955) *Endocrinology*, **56**, 305.
BRONTE-STEWART, B. AND HEPTINSTALL, R. H.—(1954) *J. Path. Bact.*, **68**, 407.
BROWN, W. D.—(1954) *Aust. J. exp. Biol. med. Sci.*, **32**, 677.
CHURCHILL, D. W., GELFANT, S., LALICH, J. J. AND ANGEVINE, D. M.—(1955) *Lab. Invest.*, **4**, 1.
DAY, A. J. AND WILKINSON, G. K.—(1956) *Aust. J. exp. Biol. med. Sci.*, **34**, 423.
Iidem AND SCHWARTZ, C. J.—(1956) *Ibid.*, **34**, 415.
FABER, M.—(1949) *Arch. Path.*, **48**, 342.
GRANT, R. T. AND ROTHSCHILD, P.—(1934) *J. Physiol.*, **81**, 265.
JACOBS, H. R.—(1949) *J. Lab. clin. Med.*, **34**, 116.
KEIDING, N. R.—(1954) *Proc. Soc. exp. Biol. N.Y.*, **86**, 390.
LOWRY, O. H., ROSEBOROUGH, N. J., FARR, A. L. AND RANDALL, R. J.—(1951) *J. biol. Chem.*, **193**, 265.
MANCINI, R. E., GARBERI, J. C. AND DE LA BALZE, F. A.—(1951) *Rev. Soc. argent. Biol.*, **27**, 285.
MENZIES, D. W. AND MILLS, K. W.—(1957) *J. Path. Bact.*, **73**, 223.
MEYER, B. J. AND VOS, A. C.—(1956) *S. Afr. J. med. Sc.*, **21**, 121.
MOON, H. D. AND RINEHART, J. F.—(1952) *Circulation*, **6**, 481.
NIELSEN, G. H. AND POULSEN, J. E.—(1954) *Dan. Med. Bull.*, **1**, 70.
PYÖRÄLÄ, K., PUNSAR, S., SEPPÄLÄ, T. AND KARLSSON, K.—(1958) *Acta Path. microbiol. scand.*, **41**, 497.
ROBB-SMITH, A. H. T.—(1954) in 'Connective Tissues' in Health and Disease'. Copenhagen (E. Munksgaard), 28.
SCHWARTZ, C. J. AND CASLEY-SMITH, J. R.—(1958) *Aust. J. exp. Biol. med. Sci.*, **36**, 117.
Idem AND GILMORE, H. R.—(1958) *Circulation*, **18**, 391.
Idem, PETERS, J. A. AND DAY, A. J.—(1958) *Aust. J. exp. Biol. med. Sci.*, **36**, 109.
TAYLOR, H. E.—(1953) *Amer. J. Path.*, **29**, 871.
Idem AND SAUNDERS, A. M.—(1957) *Ibid.*, **33**, 525.
WATSON, E. M. AND PEARCE, R. H.—(1950) *Ann. N.Y. Acad. Sci.*, **52**, 1004.
WEST, R. W. AND CLARKE, D. H.—(1938) *J. clin. Invest.*, **17**, 173.
WINZLER, R. J., DEVOR, A. W., MEHL, J. A. AND SMYTH, I. M.—(1943) *Ibid.*, **27**, 609.
ZLATKIS, A., ZAK, B. AND BOYLE, A. J.—(1953) *J. Lab. clin. Med.*, **41**, 486.

MARROW TREATMENT OF IRRADIATED DOGS*

K. A. PORTER AND N. P. COUCH

From the Department of Pathology, St. Mary's Hospital, London, W.2, and Laboratories for Surgical Research, Harvard Medical School, Boston, U.S.A.

Received for publication August 27, 1958

TREATMENT with intravenous homologous bone marrow protects mice (Lorenz, Congdon and Uphoff, 1952), rats (Fishler, Cole, Bond and Milne, 1954), hamsters (Smith, Marston, Gonshery, Alderman and Ruth, 1955), and rabbits (Porter and Murray, 1958) against the lethal effects of total body X-irradiation during the early post-irradiation period.

Similar treatment of irradiated dogs has not met with the same success (Rekers, Coulter and Warren, 1950). Use in the latter study of a dose of irradiation in the LD_{50} range may have been a major factor contributing to failure, as it is now known that supralethal doses of radiation are usually necessary to obtain permanent marrow homotransplants (Trentin, 1956 ; Gengozian and Makinodan, 1957).

In the present experiments we have studied the influence of the dose of radiation on the success of marrow homotransplantation in the dog. Survival of the animal and temporary haemopoietic transplantation were seen in some dogs after 450 r and 500 r of X-rays. Larger doses of irradiation caused death in spite of evidence of haemopoietic repopulation.

MATERIALS AND METHODS

Male mongrel dogs weighing 7·0–9·4 kg. were used as recipient animals. After being anaesthetized with intravenous Nembutal they were exposed to whole body X-irradiation under the following conditions : 250 kv.p., 15 mA, 70 cm. target distance, 0·5 mm. Cu, 0·25 mm. Sn, and 1·0 mm. Al filters, HVL=3·0 mm. Cu, 30 r/min. dose rate. Calibration was carried out with a Victoreen ionization chamber.

The marrow for post-irradiation injection was obtained from 3-weeks–2-months-old female mongrel puppies. The donors were killed by an overdose of intravenous Nembutal and under sterile conditions the marrow from all 8 long limb bones was removed and gently agitated in cold physiological saline. The suspension was pressed through a nylon filter (porosity 90 μ) to exclude coarse bone spicules and then centrifuged at 600 r.p.m. for 5 min. to remove the fat. The residue was made up to 30 ml. by adding further saline and gently mixing. Nucleated cell counts were made on every sample and each dog received an average of 3250 ($\times 10^6$) cells (748–7000). The marrow suspension was given into a leg vein 1–3 hr. following irradiation. The control irradiated dogs were each given 30 ml. of saline intravenously.

After treatment all the dogs were fed milk, cooked minced meat and Purina dog chow. For the first 3 weeks they received in addition chlortetracycline hydrochloride (Aureomycin, Lederle) 250 mg. twice daily by mouth (Furth and Coulter, 1952).

* This work was partly supported by a contract (AT-(30–1)-733) between the Atomic Energy Commission and the Peter Bent Brigham Hospital and by grants (H-2791 and H-1771) from the United States Public Health service to Harvard University.

Total leucocyte counts and differentials, platelets, haemoglobin and microhaematocrits were determined every 2 days and neutrophils bearing nuclear " drumsticks " characteristic of the female (Porter, 1957*a*) were sought in stained films.

All animals dying were necropsied.

Experimental procedure

In this experiment a total of 37 dogs was studied, divided into 5 groups as indicated in the table. Group 1 were control animals given 450 r total body X-irradiation but no bone marrow (LD_{50}/30 days = 315 r). Groups 2, 3, 4 and 5 received 450 r, 500 r, 600 r and 700 r X-irradiation respectively followed by homologous bone marrow treatment.

TABLE.—*Effect of Homologous Bone Marrow on Dogs Exposed to Total Body X-irradiation*

Group	Number of dogs	X-ray dose (r)	Treatment	Number of dogs with marrow transplants	Number of dogs surviving 30 days	Mean survival time of those dying (days)
1	8	450	None	—	0	13·0
2	8	450	Bone marrow	1	1	12·5
3	8	500	,, ,,	3	2	11·5
4	8	600	,, ,,	4	0	9·9
5	5	700	,, ,,	2	0	9·0

RESULTS

Dogs exposed to irradiation without marrow treatment

All animals in Group 1 (450 r ; no marrow) succumbed within 18 days.

Following total body irradiation there was a marked fall in the total leucocyte count which reached its lowest point at the 8th–14th day. Differential counts indicated that depression of the lymphocytes occurred more rapidly than depression of the neutrophils. The platelets fell a little more slowly, minimum values occurring from about the 12th day onwards. The haemoglobin and the packed cell volume declined gently until death.

Clinically, after a brief period of inactivity and malaise immediately after the irradiation, the dogs remained fit and ate well until about the 10th–12th day when they became listless, anorexic, pyrexial and developed bloody diarrhoea and respiratory difficulty.

Necropsy showed widespread purpura and occasional diffuse haemorrhages, the lymph nodes were enlarged and haemorrhagic, the tonsils ulcerated and there were haemorrhages into the mucosa of the gut from the stomach to the anus. In most there was non-purulent bronchopneumonic pulmonary consolidation ; in some there was evidence of generalized septicaemia.

Microscopically, although all the lymph nodes showed dilated sinuses filled with erythrocyte and haemosiderin laden phagocytes and clumps of red cells, lymphoid regeneration in the follicles and medullary cords was conspicuous. In all cases there was marked reduction in cellularity of the rib marrow, the venous sinuses were dilated and filled with red cells and occasional islands of active haemopoietic regeneration were present.

Irradiated dogs treated with bone marrow

Many of the marrow treated animals behaved in a similar fashion to those in Group 1 except that leucocyte depression occurred earlier, the mean survival

time diminished and gastro-intestinal damage became greater as the irradiation dose increased.

Ten animals, however, showed evidence of a successful marrow transplant. In these dogs (1 from Group 2, 3 from Group 3, 4 from Group 4 and 2 from Group 5) a rapid rise in the total leucocyte count began at 4–7 days and reached in those surviving about 6000 cells per c. mm. at 11 days (Fig.). At the same time typical female neutrophils appeared in the peripheral blood.

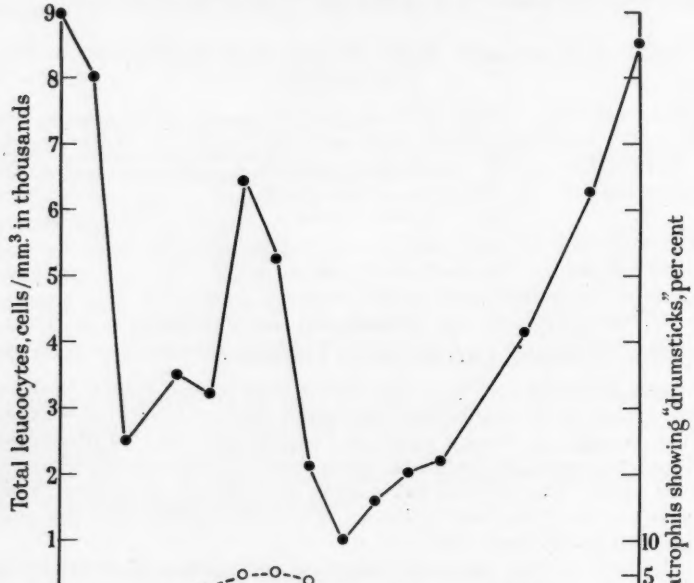


FIG.—Total leucocyte count and percentage of neutrophils showing female sex chromatin in X-irradiated male dog DR5 treated with homologous bone marrow and chlortetracycline.

●——● Total leucocytes.
○- - -○ Neutrophils showing " drumsticks ".

Seven of the dogs with marrow transplants (those from Groups 4 and 5 and 1 from Group 3), died between 11 and 20 days after irradiation, still with evidence of haemopoietic recovery and female neutrophils. At necropsy there were indications of overwhelming terminal infection and extensive denudation of the intestinal mucosa. Haemorrhage was not a feature of these cases. The rib and vertebral marrow was hyperplastic and smears from some cases showed a few female cells. Colonization of the shafts of the long bones by islands of active haemopoietic cells had occurred in several of the dogs. Lymphoid regeneration was present as in the animals of Group 1. The lungs showed no infarcts, but in one animal (in the 500 r group) which died at 20 days a few tiny calcified deposits were found

within the lumina of small branches of the pulmonary veins. Some of these showed around their margins cells looking like osteoblasts.

The 3 survivors remained healthy, had good appetites and gained weight, but by the 14th day their white cell counts had fallen to about 3000 cells, and this sharp decrease continued to about 1000 cells on days 16–18. Simultaneously, the percentage of neutrophils showing female characteristics diminished until about the 20th day when all had disappeared. From about the 18th–19th day the total white cell count in the 3 dogs rose slowly again reaching 9000 per c. mm. at about the 35th day, but female neutrophils were no longer to be found.

DISCUSSION

In these experiments transplantation of bone marrow between genetically dissimilar mongrel dogs has been achieved following doses of whole body X-irradiation well above the LD_{50}/30 day level for these animals (315 r). But many of the dogs with homologous transplants died. Death in the majority occurred early apparently from gross fluid and salt loss from the gut with a superimposed terminal infection. This heavy mortality from an intestinal syndrome, despite commencing haemopoietic recovery from a successful marrow transplant, also occurs in the rat (Fishler *et al.*, 1954) and the rabbit (Porter, 1957*b*). In the 3 dogs which survived there was evidence that the marrow homotransplant was temporary, in that donor type female neutrophils appeared as haemopoietic recovery occurred, but disappeared again later. Presumably the doses of radiation used (450 r and 500 r) briefly suppressed the immune mechanism sufficiently to allow the foreign cells to colonize the depleted host marrow spaces, but when the recipient's ability to respond returned, the transplant was destroyed. Such host rejection of the graft is often seen after sublethal irradiation and may result in a higher mortality in the marrow treated animals than in those exposed to irradiation alone (Gengozian and Makinodan, 1957).

Treatment with electrolytes, plasma and protein hydrolysates have prolonged the survival of dogs exposed to as much as 1700 r (Conard, Cronkite, Brecher and Strome, 1956). Such treatment applied to our 600 r and 700 r groups might well have reduced the early mortality and allowed survival since haemopoietic recovery from transplanted marrow was occurring in such animals. To have given the irradiation in divided dosage might also have been an advantage as has been shown in the rabbit (Porter and Murray, 1958). Recently a successful marrow homotransplant has been obtained in a dog following 1200 r given in doses of 400 r on each of 3 successive days (Ferrebee, Lochte, Jaretzk, Sahler and Thomas, 1958). The total leucocyte count in the animal described by these authors fell after initial recovery in a way identical to that seen in our dogs, but whereas in our cases female neutrophils never reappeared, in Ferrebee's case it seems they were again found at the 40th day, even though in an earlier report on this dog (Ferrebee, 1958) it was stated that " the next rise in W.B.C. was due to proliferation of this dog's own leucocytes and not that of the transplanted marrow ".

The bone marrow used in our studies was obtained from puppies largely to simplify technique as it is easy to scrape large quantities of active marrow from the shafts of the long bones of young animals. In adult dogs these bones contain mostly fat. In future work it would seem wise, however, to use foetal donor tissue in an attempt to avoid any possible late immunological reactions of transplanted tissue against host antigens (Uphoff, 1958).

In Reker's (1950) original experiments multiple bone spicules were found in the smaller pulmonary veins, and in one of our dogs a few similar calcified deposits were found. It is not possible at present to say if this is likely to be a common complication in higher animals of marrow treatment by the intravenous route. In rodents a pencil of marrow relatively free from bone spicules can be obtained with ease from the shaft of any long bone. Similar preparations from dogs inevitably contain many tiny bone fragments and osteoblasts. Filters of finer porosity than the 90 μ nylon mesh employed in our study should perhaps in future be used.

It was hoped by the use of chlortetracycline hydrochloride to reduce the incidence of overwhelming post-irradiation bacterial invasion, but like Furth, Coulter, Miller, Howland and Swisher (1953) we found little evidence of benefit.

SUMMARY

Bone marrow transplantation between mongrel dogs was attempted after several different doses of X-irradiation.

Temporary transplants of donor marrow and survival of the recipient were observed in 12·5 per cent of the animals after 450 r and in 37·5 per cent after 500 r (LD_{50}/30 days 315 r).

Although there was evidence of an initially successful marrow homograft in 50 per cent of the dogs exposed to 600 r and 40 per cent of those receiving 700 r, early death from intestinal damage occurred in all.

No permanent marrow transplants were achieved.

The authors wish to thank Dr. Francis D. Moore and Dr. J. E. Murray for their advice and encouragement, the X-ray Department of the Peter Bent Brigham Hospital and Dr. J. B. Dealy for providing the irradiation facilities and Dr. F. Lee for help with some of the radiation and marrow transplantation. Aureomycin capsules were kindly furnished by Lederle Laboratories Division, American Cyanamid Company.

REFERENCES

Conrad, R. A., Cronkite, E. P., Brecher, G. and Strome, C. P. A.—(1956) *J. appl. Physiol.*, **9**, 227.

Ferrebee, J. W.—(1958) *Blood*, **13**, Special Section—Transplantation of Bone Marrow, pp. 295 and 296.

Idem, Lochte, H. L., Jaretzki, A., Sahler, O. D. and Thomas, E. D.—(1958) *Surgery*, **43**, 516.

Fishler, M. C., Cole, L. J., Bond, V. P. and Milne, W. L.—(1954) *Amer. J. Physiol.*, **177**, 236.

Furth, F. W. and Coulter, M. P.—(1952) *Amer. J. Path.*, **28**, 25.

Iidem, Miller, R. W., Howland, J. W. and Swisher, S. J.—(1953) *J. Lab. clin. Med.*, **41**, 918.

Gengozian, N. and Makinodian, T.—(1957) *Cancer Res.*, **17**, 970.

Lorenz, E., Congdon, C. C. and Uphoff, D.—(1952) *Radiology*, **58**, 863.

Porter, K. A.—(1957*a*) *Nature*, **179**, 784.—(1957*b*) *Brit. J. exp. Path.*, **38**, 401.

Idem and Murray, J. E.—(1958) *J. nat. Cancer Inst.*, **20**, 189.

Rekers, P. E., Coulter, M. P. and Warren, S. L.—(1950) *Arch. Surg.*, **60**, 635.

Smith, W. W., Marston, R. Q., Gonshery, L., Alderman, I. M. and Ruth, H. J.—(1955) *Amer J. Physiol.*, **183**, 98.

Trentin, J. J.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **93**, 98.

Uphoff, D.—(1958) *J. nat. Cancer Inst.*, **20**, 625.

GROWTH, PURIFICATION AND MAINTENANCE OF LEPTOSPIRA ON SOLID MEDIA

L. KIRSCHNER AND L. GRAHAM

From the Department of Microbiology, Otago University Medical School, Dunedin, New Zealand

Received for publication August 28, 1958

EARLY studies of leptospira have shown that both the viability and virulence of these organisms were maintained better in semi-solid media containing corpuscular elements (small quantities of agar or fibrin) than in modified Korthof medium. Even the addition of small quantities of blood or haemoglobin to the fluid media avoided frequent transfer (every 4–6 weeks) of the many sero-types needed in leptospira reference laboratories. The frequent transfer of a large number of strains consumed much time and material, involved the risk of contamination and even confusion of the sero-types.

In recent years a higher percentage of agar was added to the above-mentioned media for certain reasons : to show that rest-phases of leptospira do exist (Woratz, 1954), to study the oxygen requirements of leptospira for growth (Lawrence, 1951), to demonstrate the growth of leptospira in various types of colonies (Cox and Larson, 1957), or to give evidence that leptospira colonies grow from single organisms (Larson and Edwards, 1958).

The purpose of our studies was to simplify the maintenance of leptospira type collections by growing these organisms as other bacteria on solid media.

We were concerned with finding a standard solid medium for maintenance of leptospira of all known sero-groups (Wolff and Broom, 1954) for long periods, easily recognizing contamination and purifying contaminated cultures from the growth on plates.

The criterion of the suitability of our solid medium consisted of obtaining a macroscopically visible spreading growth of leptospira on the plates in the form of a circle around the spotted inoculum of one loopful of organisms of a well-grown culture. In tubes which were inoculated as stab cultures with the same number of organisms single or double rings were observed.

MATERIALS AND METHODS

The first medium which gave optimum growth on plates and in tubes consisted of the following ingredients :

Basic medium A

Nutrient bacto-tryptose broth (Difco) diluted 1 in 10 in distilled water (containing 0·1 per cent bacto tryptose, 0·03 per cent beef extract and 0·05 per cent saline), 10 per cent inactivated rabbit serum, 1 per cent of 5 per cent rabbit red blood cells lysed in distilled water.

Addition of medium B.—2·6 dichlorphenol indophenol 1 : 30,000 as oxidation-reduction indicator and either vitamin B_{12} (Glaxo) 1 μg. per ml. (Babudieri and Zardi, 1956) or vitamin B_1 (thiamine) in the same quantity.

Before addition of the serum and haemoglobin medium A was adjusted to pH 7·5 and autoclaved for 15 min. at 120°. Plates and tubes closed with rubber bungs were sterilized before use in the same way. The oxidation-reduction indicator was sterilized by Seitz filtration.

The medium was poured in 10 ml. amounts in small petri-dishes (2½ in. diameter) and 3 ml. in narrow tubes (10 mm. diameter). Owing to the long incubation (1–2 weeks) at 30° and the sensitivity of leptospira to dehydration a relatively thick layer of medium (6–10 mm.) was needed in the plates. Both plates and tubes were inoculated with one standard loopful of well grown cultures containing 20,000–40,000 actively motile organisms. The plates were inoculated by spotting (4 cultures in the 4 quadrants) and the tubes as stab-cultures. They were incubated at 30°. In order to demonstrate isolated colonies of leptospira the plates were inoculated by spreading the inoculum in the usual manner. Types of all known sero-groups (Wolff and Broom, 1954) of our leptospira collection (comprising 54 strains) were used in these investigations. They were usually maintained in both the modified fluid (Korthof) and the semi-solid (Noguchi) medium containing 0·1 per cent agar. The transfer occurred every 4–6 weeks. One virulent strain of *Leptospira icterohaemorrhagiae* maintained by guinea-pig passage was also included in these studies.

RESULTS

All strains showed a characteristic growth in the tubes after incubation for 1–2 weeks. The first sign of growth was indicated by reduction of the indicator (blue to pink or red) which usually started at the bottom of the tube. After 7–10 days (rarely after 12–14 days) a heavy growth indicated by single or double rings was seen about 5–10 mm. under the surface of the medium. This confirmed the known fact that optimal conditions for the growth of leptospira are at the level of the minimal oxygen tension. The organisms are micro-aerophilic. The upper part of the medium retained its blue colour, owing to the re-oxidation effect of the oxygen present in the corked tubes. A similar observation was reported by Lawrence (1951), using a different technique (shake-cultures) in a semi-solid medium containing 0·2 per cent agar.

After longer incubation (3 weeks) the ring or rings spread deeper into the medium. Close investigation of various layers of the medium column taken out of the broken tube revealed that at the lowest part, although strongly reduced, there was no growth of leptospira. An advantage of inoculation by the stab-culture technique was early observation of contamination visible along the stab after 1–2 days' incubation. There was growth along the whole column, followed by strong reduction of the indicator (Fig. 1).

In order to check the maintenance of both viability and virulence of leptospira at various temperatures, duplicate cultures grown in properly closed tubes with rubber bungs were stored at room temperature (17°) and in the refrigerator (6°–10°). So far, after 6 months, there has been no difference in the viability of cultures in both series. However, it was noticed that in both series the indicator

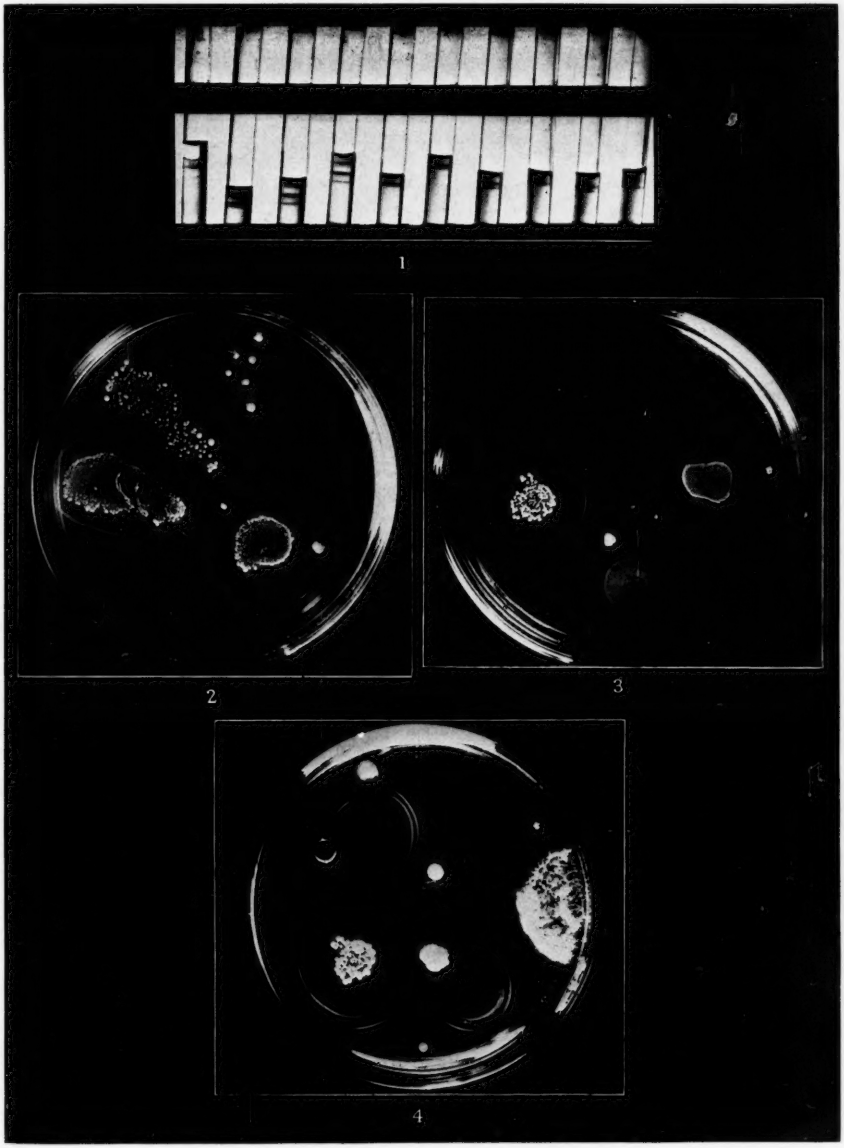
EXPLANATION OF PLATE.

FIG. 1.—Leptospira cultures in solid medium after inoculation as stab-cultures (incubation for 6 days at 30°).

FIG. 2.—Spreading growth of leptospira in circles around the spotted inoculum of one loopful of a well-grown culture, and the hazy appearance of isolated colonies around the restricted growth of contaminants.

FIG. 3.—Growth of 4 different strains after 1 week incubation at 30°. Two pure strains, one contaminated with staphylococci, the 4th strain too heavily contaminated, no growth of leptospira.

FIG. 4.—Secondary ring formation after storage of the same plate for 3 weeks at 17°.



Kirschner and Graham.

became re-oxidized. Although the red colour of the medium column had changed to blue, the rings present in the upper part remained clearly visible. A similar observation was reported by Lawrence (1951) in his semi-solid medium and he assumed that this change of the colour reflected the reduced metabolic activity of the still living organisms in the zone of the rings. This assumption was confirmed by our observations that cultures kept for longer periods (6–8 weeks) at 30° showed one or more rings in the upper part of the medium while the reduced red colour of the lower part remained unchanged.

The maintenance of the virulence of one strain of *L. icterohaemorrhagiae* available was examined by guinea-pig experiments. Suspensions of the ring-zones of stab cultures stored at room temperature and in the refrigerator were injected intraperitoneally into young 175–200 g. animals. Cultures 3 months old retained their pathogenicity much better at the lower temperature. The sensitivity of leptospira to dehydration restricted similar tests of cultures grown on plates to only 2 months. The investigations have only been proceeding for 6 months and they were done qualitatively without counting the organisms.

On plates there was a great difference in the rate of growth assessed by measuring the diameters of the macroscopically visible circles. The range of the diameters of different strains varied between 10 and 40 mm. Some strains grew over the whole plate. The growth of large numbers of organisms was observed in the medium and not on its surface. Fig. 2 clearly demonstrates the spreading growth of leptospira in circles and the hazy appearance of isolated colonies in contrast to the white colonies of contaminants. The difference in the rate of growth of leptospira and contaminants (usually staphylococci or yeasts) enabled us to purify the strains easily. The contaminants grew in circumscript colonies (Fig. 3), while a small piece of the medium cut out from the edge of the circles, usually containing a large number of leptospira only, could be transferred in Korthof or Noguchi media.

It was most remarkable that various strains of leptospira even showed growth at temperatures as low as 6°–10°. Growth was quite clearly demonstrated by the formation of new circles at room temperature (Fig. 4) and small circles appeared in the refrigerator. On both plates 4 different strains were inoculated (*L. icterohaemorrhagiae, L. pomona, L. canicola and L. australis* A). Similar observations were made with other strains used for routine investigations, *e.g. L. grippotyphosa, L. sejroe* and *L. australis* B).

There were variations in the requirements for growth promoting compounds (vitamins) by different strains in order to achieve spreading growth on plates. Some strains which were used for our routine investigations grew on plates, though in small circles (10–15 mm. diameter), containing only our basic medium mixed with rabbit or sheep serum and haemoglobin without accessory vitamins. Addition of B_{12} or B_1 vitamin (1 μg. per ml. medium) increased the rate of growth which was visible in the increased size of the circles around the inoculum. The routine strains were: Two *L. icterohaemorrhagiae* (London and Amsterdam), *L. canicola* (Amsterdam), *L. australis* A and B (Brisbane), *L. pomona* (Dunedin), *L. sejroe* and *L. grippotyphosa* (Copenhagen).

It was assumed that these strains required for the spreading growth other stimulating substances. Cytexin (Glaxo) containing B_{12} vitamin and the complex of B vitamins (thiamine, riboflavine, nicotinamide) dissolved according to instructions and added in a volume of 0·8 per cent to the solid medium gave the same results as B_{12} vitamins only.

The first heart-blood cultures of several passages of the virulent strain of *L. icterohaemorrhagiae* which was maintained in guinea-pigs showed good growth in tubes, but gave inconsistent results on plates. Recent investigations revealed that Korthof salt solution could be used as the base in place of diluted broth and 1 per cent Davis granular agar (New Zealand) in place of Difco agar. Rabbit serum, haemoglobin and indicator added to the new base and agar gave less consistent results with both virulent and non-virulent strains.

Although sheep serum is in ample supply in New Zealand it was found to be not suitable on account of the presence of antibodies, also it caused turbidity of the medium when mixed with agar.

SUMMARY

Solid media are described which gave satisfactory results in the maintenance of sero-types of all known groups of leptospira.

Frequently transferred strains in the usual fluid or semi-solid media showed, after 1–2 weeks' incubation at 30° spreading growth in the form of a circle (10–40 mm. diameter) around the spotted inoculum of one standard loopful of a well-grown culture (20,000–40,000 motile organisms). In stab-cultures growth in single or double rings 5–10 mm. under the surface was noticed.

Early growth along the whole stab indicated contamination which was accompanied by strong change in the indicator contained in the medium.

Sero-types of all known groups showed growth in tubes, but on plates the results were irregular.

The difference in the rate of growth of leptospira and contaminants on plates facilitated purification and cultures from single cell colonies of leptospira could be obtained.

Ready made solid medium, stored in corked tubes in the refrigerator could be used for at least 6 months without any change of its suitability for growth, or loss of colour of the indicator.

The material in this paper was based upon papers delivered to the New Zealand Society of Pathologists, Invercargil, May, 1958, and to the Sixth International Congress on Tropical Medicine and Malaria, Lisbon, September, 1958.

REFERENCES

Babudieri, B. and Zardi, O.—(1956) ' Proceedings 9th Italian Microbiological Congress.' Palermo, p. 227.

Cox, C. D. and Larson, A. D.—(1957) *J. Bact.*, **73**, 587.

Larson, A. D. and Edwards, C. L.—(1958) ' Bact. Proceedings ', p. 54.

Lawrence, J. J.—(1951) *Aust. J. exp. Biol. med. Sci.*, **29**, 195.

Wolff, J. W. and Broom, J. C.—(1954) *Docum. Med. geog. trop.*, **6**, 77.

Woratz, H.—(1954) *Zbl. Bakt. Orig.*, **160**, 613.

CULTIVATION OF SARCOIDOTIC TISSUE FROM LYMPH NODES AND SKIN

H. LUNDBECK, S. LÖFGREN AND H. NORDENSTAM

From the State Bacteriological Laboratory, the St. Göran's and the Serafimer Hospitals, Stockholm, Sweden

Received for publication September 2, 1958

PREVIOUS studies by the authors have dealt mainly with the clinical picture, particularly that of the incipient stage, of sarcoidosis (Löfgren and Lundbeck, 1952; Löfgren, 1953). In these studies it was demonstrated that the initial stage of the disease was characterized by bilateral, symmetrical involvement of the hilar lymph nodes, not infrequently associated with erythema nodosum. Involvement of the cervical lymph gland making possible a histopathological verification of the diagnosis was found in about 50 per cent of cases during the later part of the investigation. The material comprised 212 cases. Since then more cases of sarcoidosis have been collected, the material comprising some 400 cases at the time of writing.

Attempts at isolating bacterial and viral agents from diseased tissue by the inoculation of suitable media, animals (Löfgren and Lundbeck, 1952) and embryonated eggs (Lundbeck and Löfgren, 1952*a*) failed. It was demonstrated in a special study (Lundbeck and Löfgren, 1952*b*) that antibodies capable of fixing complement with a polysaccharide antigen from tubercle bacilli appeared in a significantly higher incidence in sera from tuberculous patients than in sera from cases of sarcoidosis. No difference in this respect was found between a control group of " normal " subjects and the sarcoidosis group.

The present paper reports some attempts to cultivate biopsy material from sarcoidotic lesions. Concurrent experiments were run to isolate cytopathogenic agents from sarcoidotic lesions with the aid of human embryonic tissue cultures.

MATERIALS AND METHODS

The clinical material for the present study comprised 35 patients with pulmonary sarcoidosis either of a recent stage with bilateral hilar lymphomas or of a chronic, generalized type (Löfgren and Lundbeck, 1952; Löfgren, 1953). Lymph nodes from the supraclavicular fossa or skin sarcoids were used in the experiments.

The materials to be investigated were removed under local xylocain anaesthesia. They were divided into 2 portions, one being used for histopathological examination and the other for the tissue culture experiment. The materials were placed in cold bovine amniotic fluid immediately after removal and were kept at 4° till the cultures were set up, generally this was done within 2—3 hr.

In all cases the histopathological examination revealed changes compatible with sarcoidosis.

The roller tube technique was used for tissue culture throughout. The specimens were cut in small fragments of about 1 cu. mm. or less. They were washed 3 times with the medium to be used, or in some instances with bovine amniotic fluid. Plasma clot cultures were then set up, 1·5 ml. medium was added to the tubes which were incubated at 35°. The medium as a rule was changed every 5 days.

The medium varied in some respects from experiment to experiment. Originally bovine amniotic fluid was used, but when it was found that this medium favoured the outgrowth of fibroblasts at the expense of other cell types, it was enriched with 5 per cent horse serum and 5 per cent bovine or human embryonic extract. The medium contained 100 units of penicillin and 50 μg. of streptomycin per ml. No difficulty in initiating growth was encountered as a rule, irrespective of which of these 2 media was used. For embryonic tissue cultures bovine amniotic fluid was employed throughout.

EXPERIMENTAL

Cultivation of sarcoidotic lymph nodes

Lymph nodes from 27 sarcoidosis patients were used for direct tissue cultivation.

In all cultures of lymph nodes 3 types of cells were easily distinguishable, lymphocytes, fibroblasts and large round cells—probably reticular cells. Within 24 hr. an abundant migration of lymphocytes was observed. The lymphocytes remained viable for a few days, then gradually disintegrated and had disappeared after 7–10 days. The " reticular " cells developed concurrently with the fibroblasts and were clearly visible on the 3rd–5th day of cultivation, occasionally even earlier. They were usually few in number and intermingled with the other cell types (Fig. 1). So far the picture was essentially the same in cultures of sarcoidosis and Hodgkin's disease lymph nodes, although the sarcoidosis cultures grew more quickly and developed a considerably greater number of cells of all kinds.

In cultures with sarcoidosis lymph node tissue a different picture gradually developed (Fig. 2). Within a few days the tissue fragments were surrounded by a carpet of fibroblasts. On a number of spots, usually near the tissue fragments, the fibroblasts became swollen and enlarged, their chromatin became coarse and the cells gradually rounded off. The changes appeared in patches. Such a patch was well delimited and walled off from the remaining fibroblasts which appeared quite normal. Fig. 2, 3 and 4 demonstrate the different stages of this development. It was often observed that the fibroblasts became club-like during the outgrowth from the tissue fragments with a thin bridge of protoplasm connecting

---

EXPLANATION OF PLATES

FIG. 1.—Pat. G. J. Lymph node culture maintained for 5 days. Fibroblasts, lymphocytes and single " reticular " cells. Normal picture. × 260.

FIG. 2.—Pat. N. N. L. Lymph node culture maintained for 5 days. Early stage of patchy degeneration in the fibroblasts. × 26.

FIG. 3.—Pat. R. A. Lymph node culture maintained for 17 days. The degenerated area is walled off and the cells within the area are completely rounded off. A few giant cells have developed. × 26.

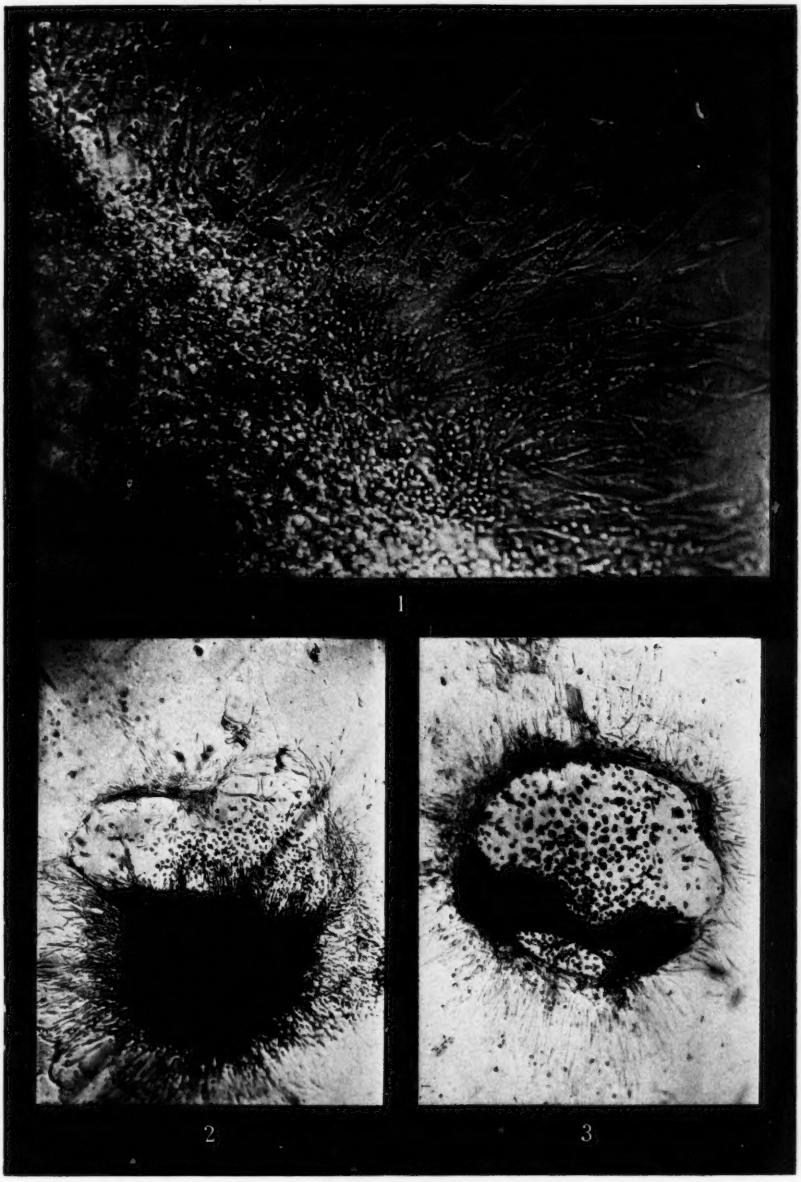
FIG. 4.—Pat. R. A. Lymph node culture maintained for 26 days. Plenty of giant cells. Margination of chromatin and beginning disintegration of the cells. × 28.

FIG. 5.—Pat. E. H. Lymph node culture, maintained for 8 days. Fibroblasts in different stages of degeneration. × 280.

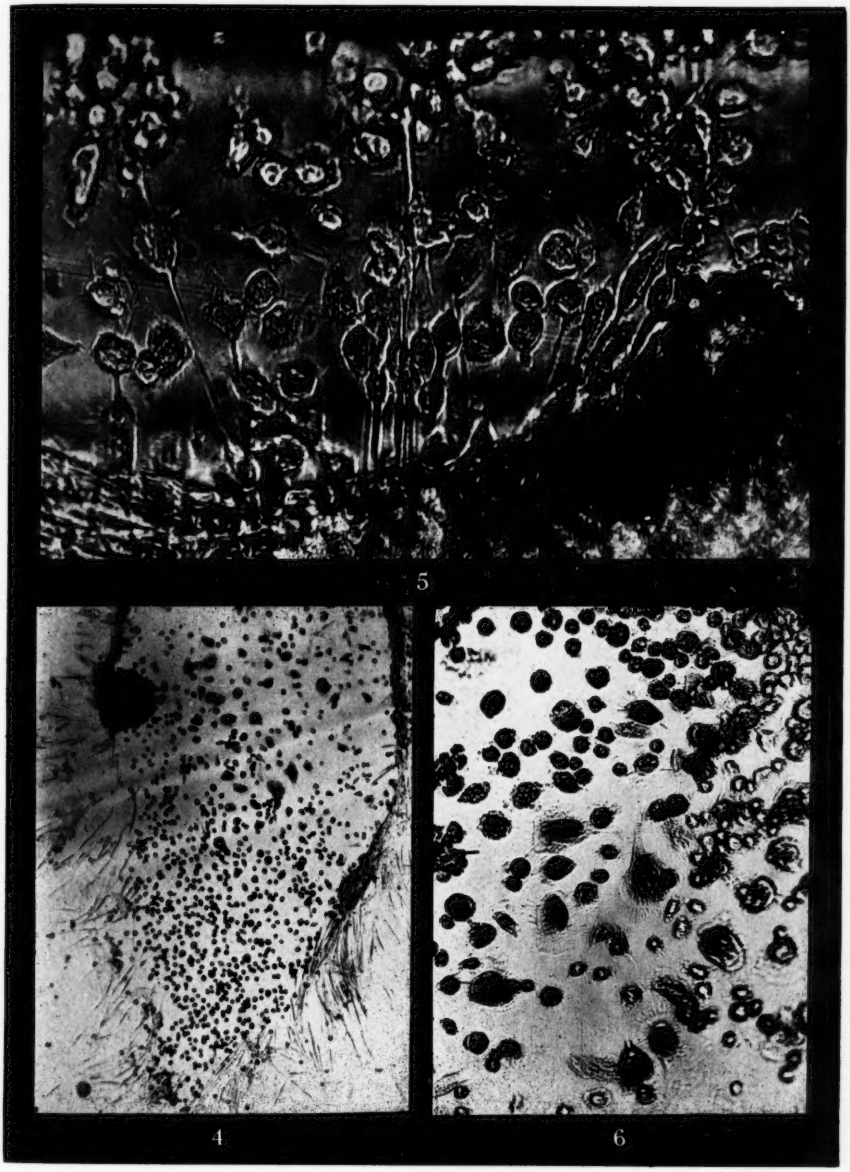
FIG. 6.—Pat. R. A. Lymph node culture maintained for 17 days. Polymorphous cells surrounded by protoplasmal processes. × 112.

FIG. 7.—Pat. N. N. L. Lymph node culture maintained for 30 days. Other data same as for Fig. 8 and 9.

FIG. 8 and 9.—Pat. R. A. Lymph node cultures maintained for 18 and 22 days respectively. Fixed in formalin, stained with Giemsa. Phase contrast. Large eosinophilic cytoplasmic inclusions visible. × 120.



Lundbeck, Löfgren and Nordenstam.

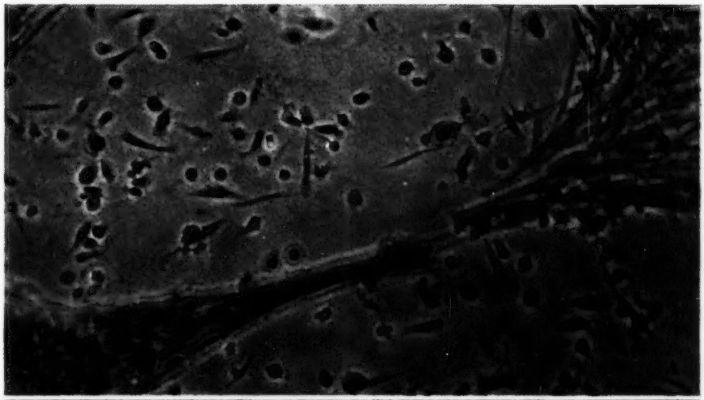


5

4

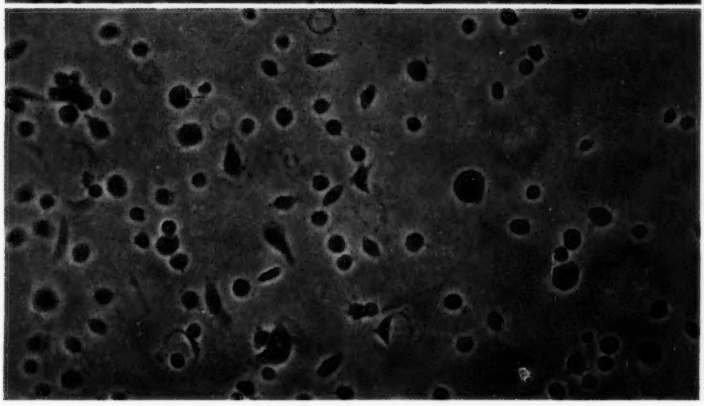
6

Lundbeck, Löfgren and Nordenstam.



9

7



8

Lundbeck, Löfgren and Nordenstam.

the rounded cell with the tissue fragment (Fig. 2 and 5). This connection was broken within 24 hr. and the cell became free. In practically all of these patches a number of giant cells developed (Fig. 4). The cell polymorphism was conspicuous (Fig. 4 and 6). The cells had a granular structure, a faint yellow colour and were often surrounded by a " corona " of protoplasmal processes (Fig. 6). The changes were fully developed in the course of a few days. The cultures were maintained for periods up to between 30 and 40 days. In the course of this time the chromatin became clumped, the cells shrank and finally disintegrated.

The events described above occurred in cultures from all sarcoidosis cases, except one. The number of typical cell formations varied, however, from case to case and from culture to culture from the same gland.

A number of cultures were fixed in formalin and slides were prepared according to the method of Cheatham (Enders and Peebles, 1954) and stained with Giemsa.

The individual cells in the cell formations described were found to be rich in chromatin and protoplasmic granular structures. All stages of development from typical fibroblasts to cells of irregular shape and to rounded cells about the size of large lymphocytes were observed. The protoplasm of the completely rounded off cells stained poorly.

In the majority of cells within these particular formations large eosinophilic cytoplasmic inclusions could be demonstrated. The inclusions were rounded or slightly irregular in shape and usually were well delimited against the protoplasm of the cells, except in cells which were completely rounded off, in these the cytoplasm was filled with amorphous red masses. In ordinary light microscopy the inclusions were slightly eosinophilic. In phase contrast microscopy the red colour became strong and conspicuous. Fig. 7, 8 and 9 are colour pictures with typical inclusions, originating from 2 different patients.

Inclusions of the kind described have been demonstrated in lymph node cultures from 5 patients out of 5 examined. The investigation is being extended to study the frequency and the nature of these inclusions.

Cultivation of skin sarcoids

Skin sarcoids from 3 patients have been cultivated. In the cultures from these materials the cell types normally yielded by skin tissue were readily recognizable. Thus bundles of cells growing out from the connective tissue, epithelial cells, and fibroblasts were consistently found. Patchy degeneration of fibroblasts similar to that described in the lymph node cultures was observed in cultures from all 3 patients. There was no significant difference in the development and appearance of the changes in the fibroblasts in lymph node and skin sarcoid cultures.

Cultivation of non-sarcoidotic tissue from lymph node and skin

Five lymph nodes from cases of Hodgkin's disease with typical histopathological changes, 3 lymph nodes with a nondescript histopathological picture, 2 lymph nodes with cancer metastases, and one naevus pigmentosus from a sarcoidosis patient were cultivated. No changes of the kind observed in the cells of the sarcoidotic tissue cultures were found in these experiments. On the whole, the picture which developed in these lymph node tissue cultures corresponded well to that described by Grand (1944 and 1949).

Experiments to isolate cytopathogenic agents in embryonic tissue cultures

Isolation experiments with the aid of embryonic tissue cultures were carried out with material from 7 cervical sarcoidotic lymph nodes and 4 cutaneous sarcoids. The materials were ground in a mortar with a few ml. of bovine amniotic fluid. The fluid in 0·1 ml. amounts was transferred to human embryonic lung tissue cultures ranging in age from 2 to 7 days. In 6 instances 2 subcultures and in 1 instance 1 subculture were initiated at various intervals after setting up the original cultures. Groups of 5 cultures were used. For passage the tissue fragments were crushed with a glass rod, loosened from the plasma film and the content of the tubes within the group was pooled. Then 0·1 ml. of this pool was transferred to each subculture tube. The cultures were maintained for periods ranging between 5 and 37 days (usually 20–23 days) and were examined every 2 days.

No cytopathogenic effect maintainable in subculture was found in any of these experiments.

Subcultures were also made by transfer of material from 7 lymph node tissue cultures to human embryonic lung tissue cultures 3–7 days old. The transfer was carried out as described above. Subcultures were performed in 3 instances through up to 4 passages. No transferable cytopathogenic effect was recorded in any of these experiments.

DISCUSSION

There seem to be two observations of interest in the present study. Firstly, a uniform picture, as far as the development of the patchy changes in the fibroblast outgrowth is concerned, has been found in 26 out of 27 sarcoidosis cases and in cultures with lymph nodes as well as with skin sarcoid tissue. No such changes of fibroblasts could be demonstrated in cultures with 11 non-sarcoidotic materials. Secondly, cytoplasmic inclusions have been demonstrated in the cells within these changed areas.

There seems to be little reason to speculate on the nature of the cytoplasmic inclusions at the present stage of the investigation. The appearance of the inclusions and their eosinophilic nature makes it probable that they consist of material alien to the cell. Whether they are simply ingested material, non-specific degeneration products, or the result of activity of an infective agent cannot be assessed on the basis of the present knowledge. Obviously any attempt at an interpretation of the significance of this finding must await the results of further investigations. Experiments to find out whether the inclusions can be maintained in passages are planned to be performed as soon as material becomes available.

The experiments to isolate an infective agent in embryonic tissue cultures are being continued. Modifications of the technique used, especially modifications designed to maintain the culture for longer periods, should be tried. Insufficient experiments have been carried out and therefore the negative outcome of the experiments cannot be considered as an indication that such cultures are insusceptible to the assumed infective agent of sarcoidosis.

The large number of sarcoidotic materials yielding the same picture in cultures makes it highly probable that these cultures represented the true tissue culture picture of sarcoidosis under the experimental conditions of this investigation.

The amount of control material, however, was too small to allow the conclusion that this picture occurs exclusively in this particular disease.

SUMMARY

Tissue from 27 lymph nodes and 3 skin sarcoids with histopathologically typical sarcoidosis were cultivated with the aid of the plasma clot technique. In cultures from all patients except one, characteristic patchy degeneration of outgrown fibroblasts was observed. No such picture was found in cultures of lymph node tissue (10 cases) and skin (1 case) with histopathologic changes other than those present in sarcoidosis.

In 5 cases out of 5 examined large eosinophilic inclusions were demonstrated in the changed fibroblasts.

A number of experiments to establish transferable cytopathogenic effects with sarcoidotic lymph node and skin tissue in human embryonic lung tissue cultures failed.

The present investigation was supported by a grant from the Swedish National Association against Tuberculosis. This paper was read at the Sarcoidosis Symposium held at the Brompton Hospital, London, in 1958.

REFERENCES

ENDERS, J. F. AND PEEBLES, T. C.—(1954) *Proc. Soc. exp. Biol. N.Y.*, **86**, 277.
GRAND, C. G.—(1944) *Ibid.*, **56**, 229.—(1949) *Cancer Res.*, **9**, 183.
LÖFGREN, S.—(1953) *Acta med. Scand.*, **145**, 424.
Idem AND LUNDBECK, H.—(1952) *Ibid.*, **142**, 259.
LUNDBECK, H. AND LÖFGREN, S.—(1952*a*) *Ibid.*, **143**, 98.—(1952*b*) *Ibid.*, **102**, 50.

A CYTOCHEMICAL STUDY OF OXIDATIVE ENZYMES IN THE PARATHYROID OXYPHIL CELL AND THEIR FUNCTIONAL SIGNIFICANCE

G. TREMBLAY* AND A. G. E. PEARSE

From the Department of Pathology, Postgraduate Medical School of London, Du Cane Road, London, W.12

Received for publication September 5, 1958

ALTHOUGH 60 years have elapsed since the oxyphil cells of the parathyroid gland were described by Welsh (1898) their nature has remained an enigma (Trier, 1958) and their physiological significance has been the object of conflicting views. It is generally believed that they represent involuted inactive chief cells (Castleman and Mallory, 1935; Cowdry, 1938; Castleman, 1952; Krook, 1957). This view was supported by Hamperl (1950) who considered that they arose from a peculiar degeneration of the parenchymatous cells and were analogous to other granular eosinophilic cells (onkocytes) found in the thyroid, pituitary, adrenal, salivary gland and in various epithelial tissues.

On the other hand, Warren and Morgan (1935) have suggested that in varying degree all parathyroid cells, including the mature oxyphil form, are capable of elaborating parathyroid hormone. Other authors have mentioned the possibility that the oxyphil cells may be active and functionally distinct from the principal cells (Greep, 1955; Black and Ackerman, 1950) and Rucard (1949), on the basis of an extensive histological study of the parathyroid, concluded that the oxyphil cells possess a secretory activity.

Newer histochemical techniques should help in elucidating the true nature of the oxyphil cells, and Christie (1955), showing by Baker's acid haematein method that oxyphil cells contained a large amount of phospholipid, postulated that they were functionally active. More recently, Trier (1958), has shown by means of the electron microscope that the oxyphil cells of the monkey parathyroid are "packed" with mitochondria. However, nothing is known of the enzymatic content of these cells. The present report concerns a study of oxidative enzymes in the oxyphil cells of the monkey parathyroid, using cytochemical techniques.

MATERIAL AND METHODS

Since human parathyroid glands taken at autopsy were found to be generally unsuitable due to extensive post mortem damage to cytoplasmic constituents and oxidative enzymes, monkey parathyroids (*Macaca irus*) were used.

Preliminary studies showed that the parathyroid of the adult *Macaca irus* is similar to that

* Fellow of the Canadian Cancer Society. Permanent address: Hotel-Dieu Hospital, Montreal, Canada.

of the *Macaca mulatta* (Cowdry and Scott, 1936; Baker, 1942; Trier, 1958) and contains both principal and oxyphil cells. Material from adult male and female animals and in two instances from young male animals was used. In addition, 2 parathyroids from a man aged 64 who died of peritonitis were studied 4 hr. after death. The animals were anaesthetized with nitrous oxide and pentobarbitone sodium. While the animals were under anaesthesia the two external parathyroids were excised and embedded in a piece of liver to avoid distortion during sectioning. Frozen sections 8 μ thick were cut in a remotely controlled cold microtome (cryostat). Diphosphopyridine nucleotide diaphorase (DPND), triphosphopyridine nucleotide diaphorase (TPND), succinic dehydrogenase, β-hydroxybutyric dehydrogenase and glucose-6-phosphate dehydrogenase were determined by the technique of Pearse (1957), Hess, Scarpelli and Pearse (1958), Scarpelli, Hess and Pearse (1958) using 3,5-diphenyl-2-(4,5-dimethylthiazol-2-yl) tetrazolium bromide (MTT) as the final electron acceptor. On the human parathyroid gland only DPN and TPN diaphorase were studied. With these techniques the sites of enzymatic activity appear as small formazan deposits within the mitochondria (Pearse and Scarpelli, 1958). Identification of the oxyphil cells was accomplished as follows: pictures were taken of selected fields after running the various enzyme reactions. The metal-formazan dye which is the product of these reactions was removed from the sections by treatment with alcohol and the sections were then re-stained with haematoxylin and eosin. The same fields were identified and photographed. Identification of the oxyphil cells was readily assured by comparison of the two sets of pictures.

RESULTS

The oxyphil cells of the monkey parathyroid contained a high activity of DPNH and TPNH diaphorase (Fig. 1, 3, 5) as well as of succinic, β-hydroxybutyric and glucose-6-phosphate dehydrogenases. The most intense reaction was observed in the case of the DPNH diaphorase. In the principal cells, on the contrary, the enzymatic activity of these dehydrogenases and diaphorases was weak. This striking contrast in the intensity of the enzymatic reaction between the two types of cells was evident even at low magnification (Fig. 1). In the parathyroid of young animals in which no oxyphil cells were seen the staining reaction was uniformly weak throughout the gland (Fig. 2).

The oxyphil cells contain numerous mitochondria evenly distributed in the cytoplasm (Fig. 7). In the principal cells, on the other hand, the mitochondria are more scarce, frequently being aggregated at the vascular pole and orientated parallel to the long axis of the cells (Fig. 8).

No sex difference in the enzymatic pattern of the oxyphil cell was found.

The oxyphil cell in the human parathyroid examined for DPNH and TPNH diaphorases showed a high enzymatic activity while the principal cells exhibited a weak reaction (Fig. 6).

DISCUSSION

Baker, in 1942, reported that some of the oxyphil cells in the macaque parathyroid contained numerous mitochondria and recently Trier (1958), using the electron microscope, showed that the oxyphil cells contain more mitochondria than the principal cells. The present study confirms this by means of cytochemical techniques and, in addition, shows that they exhibit a high activity for various dehydrogenases associated with the tricarboxylic acid cycle, fatty acid oxidation, glucose metabolism and electron transport in pyridine nucleotide-linked reactions. These results suggest that oxyphil cells are not involuted cells but highly active elements of the parathyroid gland.

Although the oxyphil cells contain more mitochondria and show a greater activity of oxidative enzymes than the principal cells it does not necessarily follow that their secretory activity is greater. It may simply mean that the oxyphil cells need a greater energy supply than the principal cells to perform their function. A similar difference has been observed between the chief cells and the parietal cells of the stomach which possess a different function and show a different enzymatic pattern (Hess *et al.*, 1958). Except for DPN diaphorase, the 4 dehydrogenases studied in the oxyphil cells were equally active and no conclusion can be drawn as to the predominant oxidative pathway in these cells.

Previously we reported (Pearse and Tremblay, 1958) a relation between leucine aminopeptidase activity and parathyroid function. The method of Nachlas, Crawford and Seligman (1957) showed considerable leucine aminopeptidase activity in the principal cells of the monkey parathyroid. Since the oxyphil cells occur as isolated cells or groups of 2 or 3 cells among the principal cells it has not been possible to determine with certainty whether they contain leucine aminopeptidase.

It has been firmly established that the principal cells possess an endocrine secretory activity since they are the only cells present in some animals (Rosof, 1934 ; De Robertis, 1940 ; Foster, 1943), in primary hyperplasia of the human parathyroid glands (Castleman, 1952) and in some tumours of the parathyroid with symptoms of hyperparathyroidism (Black and Ackerman, 1950). While the present observations indicate that the oxyphil cells are also metabolically active, their function has not yet been ascertained. However, in this regard it is interesting to point to recent evidence that the parathyroid gland may possess several physiological effects (Greep and Kenny, 1955). Furthermore, the parathyroid hormone has not yet been successfully purified and the highly active preparation obtained by Rasmussen and Westall (1957), which has been characterized as a mixture of polypeptides, may contain more than one hormone. Therefore, it seems reasonable to suggest that the oxyphil cells may secrete a hormone whose target organ and physiological action remain as yet obscure.

Although oxyphil cells have been reported in the parathyroid of children (Marine, 1932 ; Morgan, 1936 ; Gilmour, 1939) it is more generally accepted that these cells appear at puberty and increase with age (Castleman and Mallory, 1935 ; Castleman, 1952). This fact has been interpreted by Castleman and Mallory (1935) as an indication of the degenerative character of these cells. However as pointed out by Black and Ackerman (1950) it may signify that with advancing age there is an increased need for the oxyphil cells.

A point of additional interest is the distribution of oxyphil cells in the parathyroid gland of various animals. Oxyphil cells are present in the parathyroid gland of the monkey (Cowdry and Scott, 1936 ; Baker, 1942 ; Trier, 1958) of the cow and steer (Levine, 1928) and of the horse (Rucart, 1949). Occasional oxyphil cells have also been reported in the gland of the dog, particularly in older animals (Krook, 1957) and of the rabbit (Drake, Albright and Castleman, 1937). However, they have never been found in the parathyroid gland of the guinea-pig (Rucart, 1949), rat (Rosof, 1934 ; De Robertis, 1940) and mouse (Foster, 1943). The significance of this, although not yet evident, may mean that the oxyphil cells secrete a substance which is necessary in some animals but not in others.

Although there have been reports to the contrary (Sommers and Young, 1952), it is generally believed that parathyroid adenomata composed wholly of oxyphil

cells are hormonally inactive. However, since this has been based on the absence of clinical symptoms it may be that gross disturbances of the physiological function or functions subserved by the oxyphil cell do not give rise to symptoms.

SUMMARY

The activity of succinic, β-hydroxybutyric and glucose-6-phosphate dehydrogenase and of DPNH and TPNH diaphorase in the monkey parathyroid has been studied with cytochemical techniques. In addition, fresh human parathyroids were examined for DPNH and TPNH diaphorase activity.

The oxyphil cells showed a high activity of all dehydrogenases and of both diaphorases. In the principal cells the enzymatic reactions were weak.

The physiological significance of these findings is discussed. It is suggested that the oxyphil cells are not degenerate, but are active secretory elements of the parathyroid gland.

We wish to thank Dr. S. A. Keeble and Dr. A. C. Laursen of the Glaxo Laboratories for making available the animals used in these experiments. The photomicrographs were taken by Mr. W. Brackenbury.

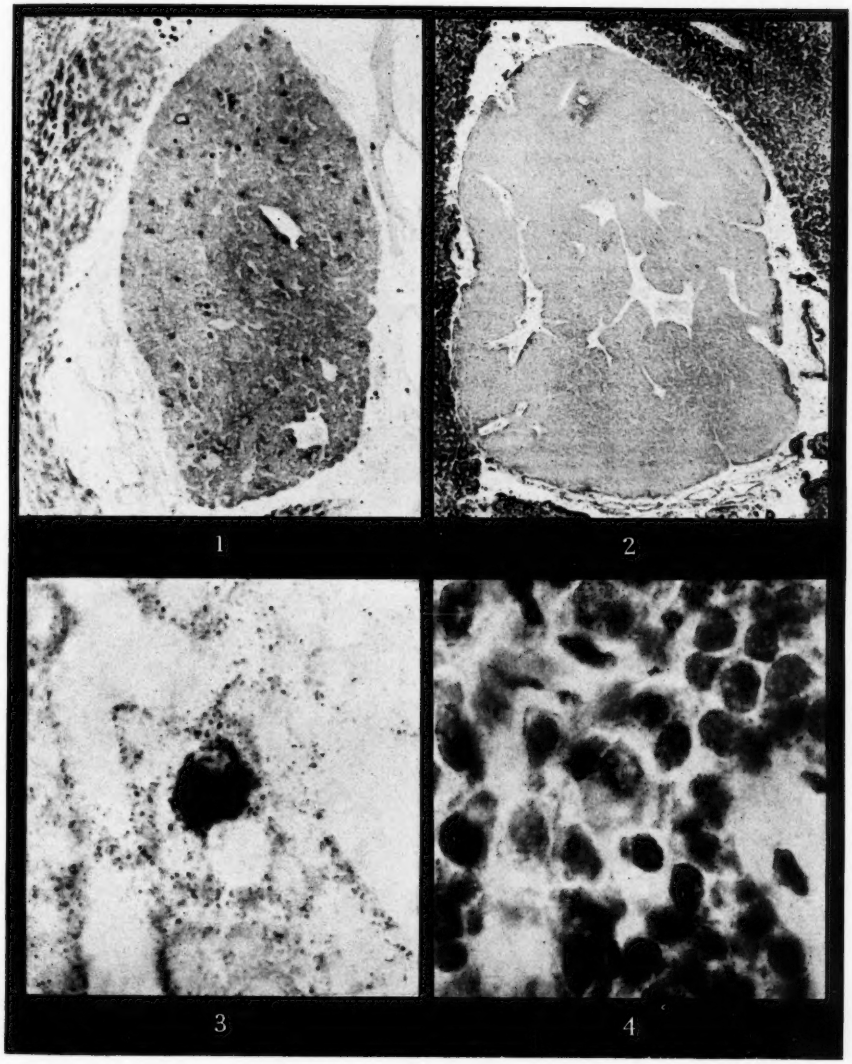
REFERENCES

Baker, B. L.—(1942) *Anat. Rec.*, **83**, 47.
Black, B. K. and Ackerman, L. V.—(1950) *Cancer*, **3**, 415.
Castleman, B.—(1952) ' Atlas of Tumour Pathology. Tumours of the Parathyroid Glands '. Washington (A.F.I.P.), Sect. IV, Fasc. 15, p. 74.
Idem and Mallory, T. B.—(1935) *Amer. J. Path.*, **11**, 1.
Cowdry, E. V.—(1938) ' A Textbook of Histology '. London (Henry Kimpton), p. 210.
Idem and Scott, C. H.—(1936) *Arch. Path.*, **22**, 1.
Christie, A. C.—(1955) *J. clin. Path.*, **8**, 302.
De Robertis, E.—(1940) *Anat. Rec.*, **78**, 473.
Drake, T. C., Albright, F. and Castleman, B.—(1937) *J. clin. Invest.*, **16**, 203.
Foster, C. L.—(1943) *J. Endocrin.*, **3**, 244.
Gilmour, J. L.—(1939) *J. Path. Bact.*, **48**, 187.
Greep, R. O.—(1955) In ' Histology '. London (Churchill Ltd.), p. 822.
Idem and Kenny, A. D.—(1955) In ' The Hormones ', edited by G. Pincus and K. V. Thimann. New York (Academic Press Inc.), Vol. III, p. 153.
Hamperl, H.—(1950) *Arch. Path.*, **49**, 563.
Hess, R., Scarpelli, D. G. and Pearse, A. G. E.—(1958) *Nature*, **181**, 1531.
Krook, L.—(1957) *Acta path. microb. scand.*, Suppl. 122, Vol. 41, p. 88.
Levine, M.—(1928) *Anat. Rec.*, **39**, 293.
Marine, D.—(1932) In ' Special Cytology ', edited by E. V. Cowdry, 2nd Ed. New York (Paul B. Hoeber), Vol. II, p. 827.
Morgan, J. R. E.—(1936) *Arch. Path.*, **21**, 10.
Nachlas, M. M., Crawford, D. T. and Seligman, A. M.—(1957) *J. Histochem. Cytochem.*, **5**, 264.
Pearse, A. G. E.—(1957) *Ibid.*, **5**, 515.
Idem and Scarpelli, D. G.—(1958) *Nature*, **181**, 702.
Idem and Tremblay, G.—(1958) *Ibid.*, **181**, 1532.
Rasmussen, H. and Westall, R. G.—(1957) *Biochem. J.*, **67**, 658.
Rosof, J. A.—(1934) *J. exp. Zool.*, **68**, 121.

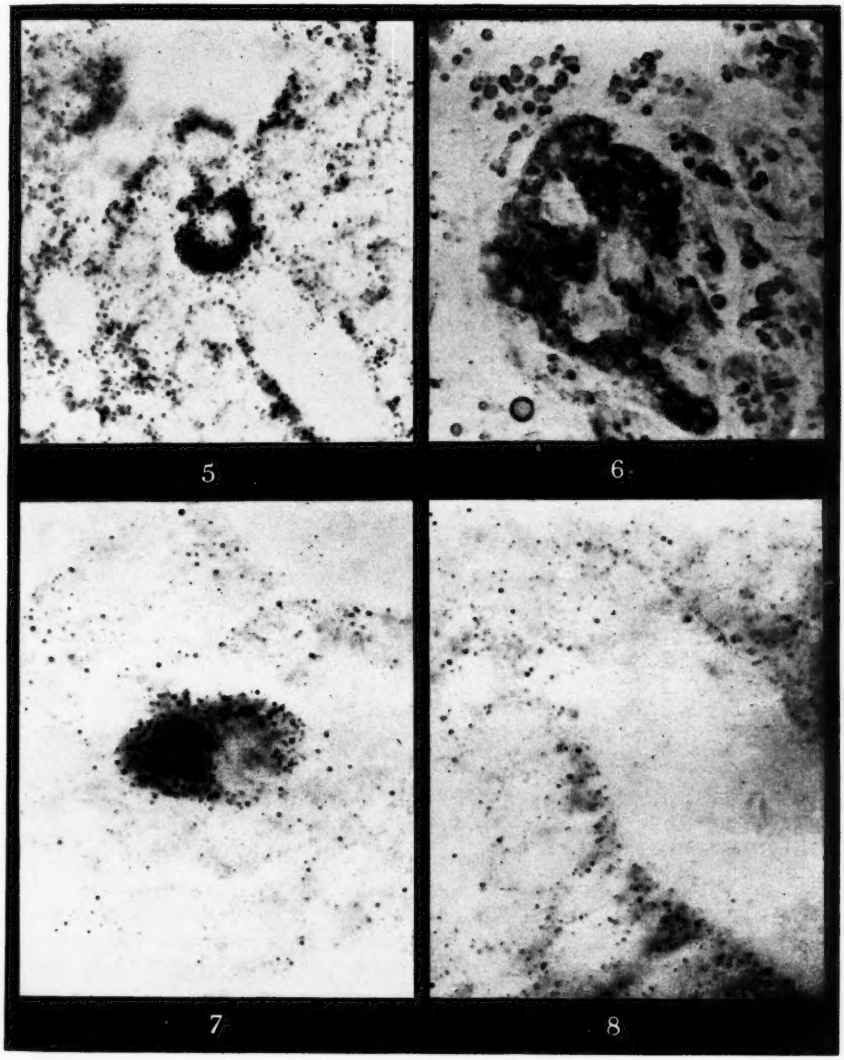
RUCART, G.—(1949) *Arch. Anat. micr. Morph. xep.*, **38**, 1.
SCARPELLI, D. G., HESS, R. AND PEARSE, A. G. E.—(1958, *J. biophys. biochem. Cytol.* (in press).
SOMMERS, S. C. AND YOUNG, L.—(1952) *Amer. J. Path.*, **28**, 673.
TRIER, U. S.—(1958) *J. biophys. biochem. Cytol.*, **4**, 13.
WARREN, S. AND MORGAN, J. R. E.—(1935) *Arch. Path.*, **20**, 823.
WELSH, D. A.—(1898) *J. Anat. Lond.*, **32**, 380.

EXPLANATION OF PLATES

FIG. 1.—DPNH diaphorase activity in parathyroid gland of an adult male monkey. Scattered oxyphil cells are strongly reactive. The principal cells show weak activity. × 54.
FIG. 2.—DPNH diaphorase activity in parathyroid of a young male monkey. No oxyphil cells are seen and the enzymatic reaction is diffusely weak throughout the gland. × 48.
FIG. 3.—DPND reaction in monkey parathyroid gland. An isolated oxyphil cell shows intense activity contrasting with the weak reaction in the principal cells. The nuclei contain no enzyme and appear as clear round spaces. × 900.
FIG. 4.—The same section as in Fig. 3 stained with haematoxylin and eosin. The strongly reactive cell is an oxyphil. × 900.
FIG. 5.—TPNH diaphorase activity in parathyroid of an adult monkey. An isolated oxyphil cell is strongly reactive while the principal cells are weakly positive. × 900.
FIG. 6.—DPNH reaction in a human parathyroid gland. A group of oxyphil cells react strongly. The oxyphil cells and the surrounding principal cells show early post mortem changes. × 900.
FIG. 7.—DPND reaction in a portion of parathyroid containing a single oxyphil cell. The mitochondria demonstrated by the formazan deposits are numerous and distributed throughout the cytoplasm. × 1520.
FIG. 8.—DPNH activity in principal cells. The mitochondria are less numerous than in the oxyphil cells and frequently aggregated at the vascular pole of the cells. × 1520.



Tremblay and Pearse.



5 6

7 8

Tremblay and Pearse.

THE FUNCTION OF TRYPTOPHAN IN THE ADSORPTION OF A BACTERIOPHAGE

P. FILDES AND D. KAY

From the British Empire Cancer Campaign Virus Unit, Sir William Dunn School of Pathology, Oxford

Received for publication September 9, 1958

FILDES and KAY (1957) described a phage isolated from sewage which existed in the form of 2 mutants, one "tryp$^+$" differing from the other "tryp$^-$" in requiring the addition of tryptophan for adsorption to bacteria.

Further study has shown that the 2 mutants have the same relationship to tryptophan in the course of several other reactions in which they can take part. For instance, adsorption of the tryp$^+$ mutant to mineral particles or to receptor particles and its inactivation by papain are dependent on the presence of free tryptophan. Further, its susceptibility to heat and to inactivation by antiphage serum are partially dependent. The tryp$^-$ mutant is not dependent on tryptophan, although some of these reactions are accelerated by it.

TECHNIQUE

The technique in general follows that of Fildes and Kay (1957). A few protocols are given to illustrate the procedure. The chief difference is that a *Bact. coli* 518 (3) is now used for convenience as the lawn for plate counts.

The phages used in the present work were a tryp$^+$ suspension with a tryp$^+$: tryp$^-$ ratio of $1 \times 10^4 : 1$, and a tryp$^-$ with a tryp$^+$: tryp$^-$ ratio of $1 : 2{\cdot}3 \times 10^3$ (Fildes and Kay, 1957). It will be noted that neither mutant was very highly homogeneous and a certain degree of overlap in their behaviour towards tryptophan might be expected.

EXPERIMENTAL

Adsorption of Phage 3 *to Bacteria*

The general results in the previous paper have been confirmed.

Throughout the literature (Boyd, 1956) the term "adsorption" is used to denote the attachment of phages to bacteria. In the strict sense this implies a reversible attachment brought about by electrostatic bonds between phage and bacterium. In the case of phage 3 however no reversible attachment can be demonstrated and in particular no attachment at all, under the most favourable conditions, at 0°. Electrostatic adsorptions should not be affected by temperature at this level.

We assume that secondary anatomical changes in the phage tail tip follow so rapidly after contact at temperatures above 0° that no recovery of an intact phage can be made.

This appears to be a difference between phage 3 and the T series phage T4, while other differences are referred to later.

Adsorption of Phage 3 to Inanimate Particles

It is well known that Puck, Garen and Cline (1951) found that the tryptophan requiring phage T4 was adsorbed on sintered glass in the presence of tryptophan, but not in its absence. The action of tryptophan was not a co-factor in some enzymic process. because de-adsorption followed treatment with tryptophan-free diluent.

In our hands the present phage did not adsorb on finely powdered Ballotini beads to a demonstrable extent in 4 hr. whether tryptophan was present or not. We have also failed to observe adsorption on esterified cellulose or acid washed " Supercel ".

On the other hand kaolin (B.D.H. " washed with acids ") was active and its activity was precisely similar to the activity of a susceptible bacterium in that it adsorbed tryp$^+$ phage only in the presence of free tryptophan and a tryp$^-$ phage whether tryptophan was present or not.

Experiment 1.—*Effect of tryptophan on adsorption of tryp$^+$ and tryp$^-$ phage by kaolin*

The kaolin suspension was made by suspending 1·8 g. kaolin in 20 ml. plain buffer (*i.e.* not containing albumin) and heating to 60° for 1½ hr. After standing on the bench for a few minutes some of the supernatant was taken off and its opacity adjusted to 0·320 in the Spekker. In the adsorption experiment this underwent dilution 1 in 4.

Both phages were used at approximately 5×10^5 phages/ml. in buffer-albumin. Tryptophan was 0·0004 M in plain buffer. The experiment was set up as in Table I :

TABLE I

(Vols. in ml.)

Tube	Plain buffer	Tryptophan	Kaolin	Phage
1	2·5	2·5	0	tryp$^+$ 5·0
2	0	2·5	2·5	5·0
3	2·5	0	2·5	5·0
4	2·5	2·5	0	tryp$^-$ 5·0
5	0	2·5	2·5	5·0
6	2·5	0	2·5	5·0

The tubes were held at rest at 37°. Samples were taken from time to time, diluted 1 in 100 in buffer-albumin and 4 drops (0·1 ml.) deposited on a lawn of *coli* 518 (3). Counts were made after 4 hr. Fig. 1 shows the result.

Electron micrographs showed " specific " behaviour of adsorption by the tail tip.

This irreversible adsorption of phage 3 on kaolin compares with the reversible adsorption of phage T4 on glass described by Puck *et al.* (1951).

Adsorption of the tryp$^+$ phage also took place on Palygorskite in the presence of tryptophan (Fig. 2). It will be noted that the phage heads were not deflated as is the case on adsorption to bacteria. Palygorskite is a very suitable mineral for electron microscopy because it, and kaolin also, breaks down to give particles of convenient size and electron transparency to enable search for adsorbed phage particles to be successful.

Adsorption of Phage 3 to Bacterial Receptors

It is generally thought that the adsorption of a phage to a bacterium is due to a contact of the phage tail tip with a specific formation on the bacterial surface. These "receptor" substances can be extracted from the bacterium and will combine with the phage in the test tube and inactivate it under the same conditions as does the whole bacterium (Kay, 1955).

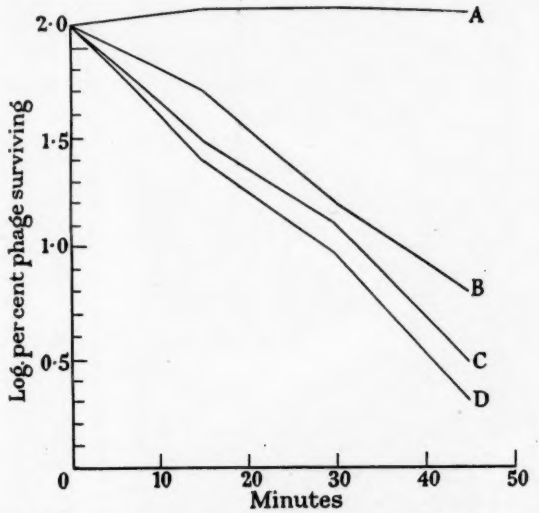


FIG. 1.—Effect of tryptophan on the adsorption of Phage 3 to kaolin.

A Tryp$^+$ phage without tryptophan.
B " " plus "
C Tryp$^-$ phage without tryptophan.
D " " plus "

In the case of phage 3, also, receptors extracted from susceptible bacteria and purified up to the acid precipitation stage (Kay, 1955) adsorbed phage under the same conditions as did the whole bacteria. Bacteria of the same species which did not adsorb the phages, did not yield receptor preparations. Fig. 4 shows the effect of the receptors from *coli* 1474. The effect is the same as that of the whole organism.

The receptor preparations are, of course, crude. They are particulate under the electron microscope and can be deposited at high speed. Indeed they may in these experiments mainly be shreds of bacterial coats since much the same result may be obtained by extracting kaolin in plain buffer at 60° for 1½ hr. The crystal clear supernatant from the angle centrifuge adsorbed phage 3, but a further centrifuging at high speed removed the activity, which the electron microscope showed to consist of minute crystals of kaolin.

Inactivation of Phage by Heat

Cheng (1956) stated that the tryptophan-requiring T4 phage was unaffected for 1 hr. by a temperature of 53·5°, but was destroyed in the presence of tryptophan.

Our experiments were carried out to test the thermal stability of phage 3 tryp$^+$, equivalent to the T4 phage, and of the mutant tryp$^-$.

As a preliminary both phages were heated for 6 hr. at 0°, 18°, 25°, 37°, 45° and 50°. Samples were removed from time to time and plated. Both phages were unaffected for 6 hr. at temperatures below 45°, but both were inactivated at 45°. Experiment 2 shows the procedure in an exposure lasting 50 min.

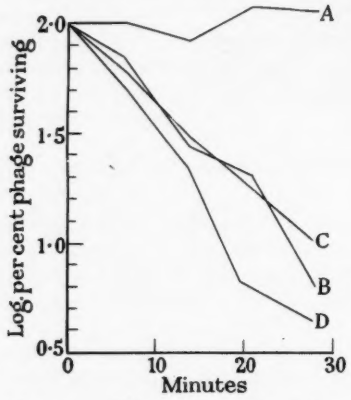


FIG. 4.—Adsorption of Phage 3 to receptors from *coli* 1474.

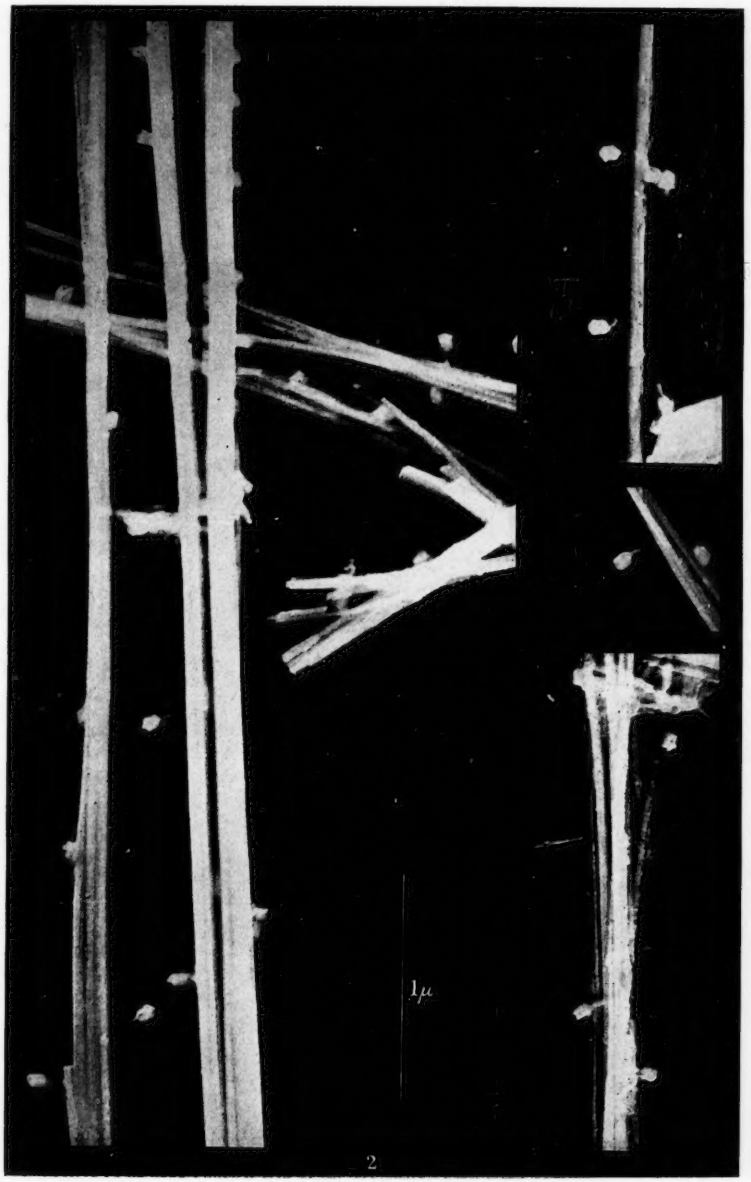
A Tryp$^+$ phage without tryptophan.
B " " + "
C Tryp$^-$ phage without tryptophan.
D " " + "

Experiment 2.—Effect of tryptophan on the heat lability of tryp$^+$ and tryp$^-$ phages

The two phages were at approximately 2·5 × 10^5/ml. in buffer-albumin each with and without 0·0001 M DL-tryptophan. A sample was heated at 37° and showed no alteration in 50 min., the remainder was heated at 45°. Samples were removed every 10 min., diluted 1/100 in buffer-albumin and plated (4 drops). The input of phage was averaged from the counts at 37°. The other counts were plotted as log per cent of the input surviving against time (Fig. 5). In 50 min. at 45° both phages showed inactivation, the tryp$^-$ to a greater extent than the tryp$^+$. On addition of tryptophan, however, the tryp$^-$ phage showed no greater inactivation, whereas the tryp$^+$ phage showed more than 3 times as much. Tryptophan thus governed the behaviour of these 2 phages towards heat as it did towards adsorption.

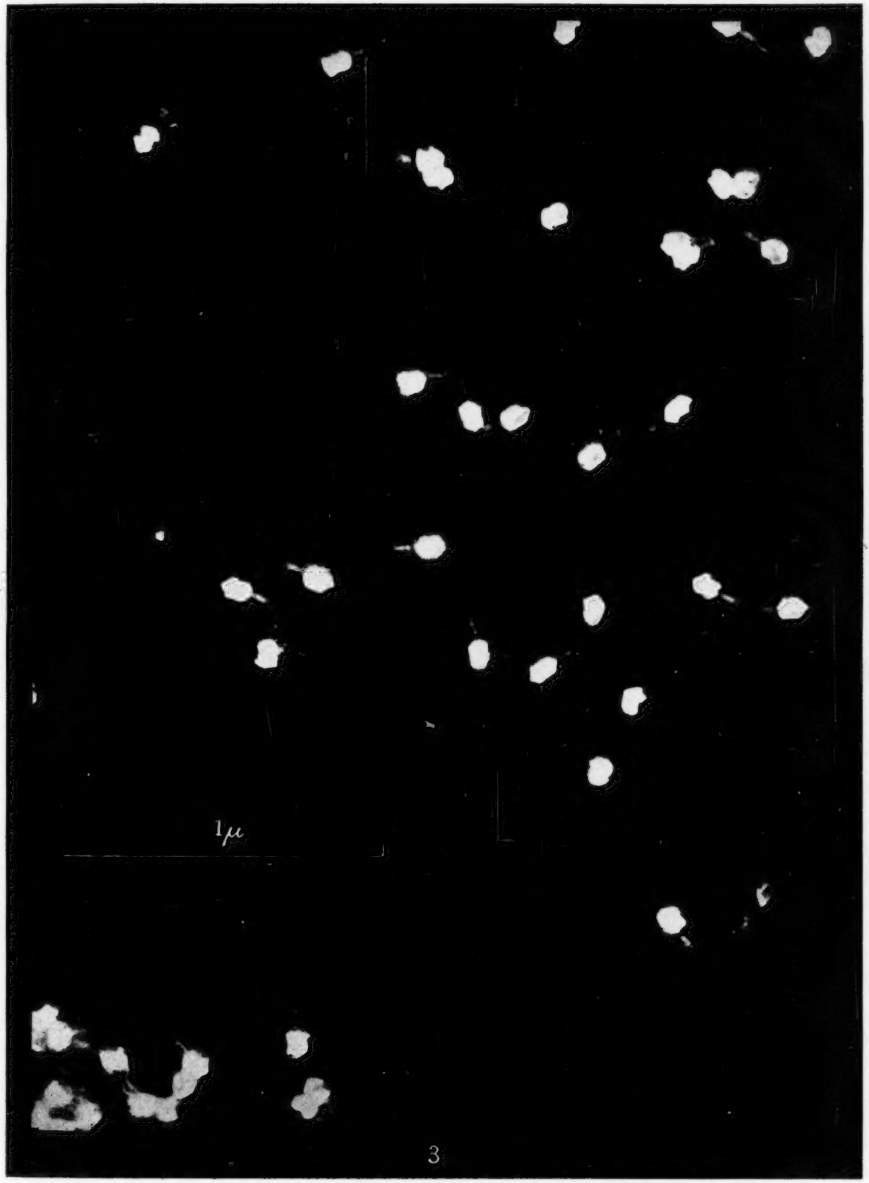
EXPLANATION OF PLATES.

FIG. 2.—Phage 3 tryp$^+$ adsorbed to Palygorskite in presence of tryptophan.
FIG. 3.—Phage 3 tryp$^+$ inactivated by heating at 45° for 1 hr. with tryptophan.



2

Fildes and Kay.



Fildes and Kay.

Cheng (1956) has already described anatomical changes in the tail tip of phage T4 resulting from heating to 53°. Fig. 3 shows similar effects on the tryp$^+$ phage with tryptophan at 45°. The hollow cylindrical tail has lost its terminal fibres and has contracted lengthwise revealing the central tail-spike.

Inactivation of Phage 3 *by Papain*

We came to test papain because Kalmanson and Bronfenbrenner (1943) claimed to have reactivated with it phage which had been treated with antiphage serum.

When we designed an experiment on these lines we found that phage 3, unlike Kalmanson and Bronfenbrenner's phage and our phage 1, was highly susceptible

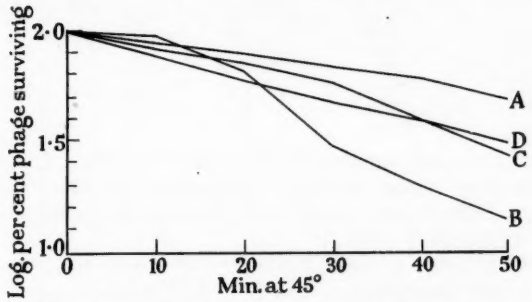


FIG. 5.—Effect of tryptophan on heating Phage 3 tryp$^+$ and tryp$^-$ at 45°.

A Tryp$^+$ phage without tryptophan.
B " " plus "
C Tryp$^-$ phage without tryptophan.
D " " plus "

to the direct action of papain. Our papain preparation directly inactivated our tryp$^-$ phage, but our tryp$^+$ only after addition of tryptophan.

Preparation of papain extract

The papain used in this work was a crude preparation from the finely powdered "Papain (B.D.H.)". Papain (4 g.) was ground with 100 ml. phosphate buffer at pH 7·6 and heated for 1½ hr. at 37°. Phosphate buffer, 0·033 M (80 ml.) and M-cysteine hydrochloride (20 ml.) were then added. The pH was adjusted to 7·6 with NaOH (about 27 ml. N/1), the whole was warmed in water at 37° for 1 hr. and then filtered after cooling. The digest was then dialysed for 3 days against 1 litre buffer, this being changed once a day. Thymol was added both inside and outside the dialysis tube. The preparation being at the required pH and buffer concentration could be used directly in an experiment at a dilution 1/4. With growing bacteria a 1/4 dilution of saturated thymol is inhibitory, but in an adsorption experiment with phage no ill effects were observed.

Experiment 3.—*The action of papain on tryp$^+$ and tryp$^-$ phages*

Table II shows the design of this experiment. The dialysed papain preparation was activated by addition of 0·1 M-cysteine making 0·025 M final concentration.

The tubes were held at 37° for 1 hr., then diluted 1 in 100 in buffer-albumin and plated (0·1 ml.). The papain was heated for 30 min. at 100°.

TABLE II

Tube	Buffer (ml.)	Phage 5×10^5 ml. approx. tryp$^+$	Papain + 0·1 M-cysteine: Unheated (ml.)	Papain + 0·1 M-cysteine: Heated (ml.)	DL-tryptophan M/2500 (ml.)	Counts
1	5·0	5·0	—	—	—	290
2	2·5	5·0	2·5	—	—	294
3	0	5·0	2·5	—	2·5	65
4	2·5	5·0	—	2·5	—	280
5	0	5·0	—	2·5	2·5	292
		tryp$^-$				
6	5·0	5·0	—	—	—	288
7	2·5	5·0	2·5	—	—	40
8	0	5·0	2·5	—	2·5	13
9	2·5	5·0	—	2·5	—	271
10	0	5·0	—	2·5	2·5	280

It will be seen that heated papain had no action on either phage : that papain had no action on tryp$^+$ phage without tryptophan but inactivated 78 per cent with tryptophan ; that papain inactivated 87 per cent of tryp$^-$ phages without tryptophan and 96 per cent with.

The activity of papain on these 2 phages had therefore the same relationship to tryptophan as had adsorption.

The mechanism of the action of papain on phage 3

It was assumed at first that the action of papain on phage 3 was due to its protease activity but this view had to be abandoned. As shown by Kimmel and Smith (1954) the protease of papain is most active in the presence of cysteine to convert it to the -SH form and with the addition of citrate or ethylenediaminetetra-acetic acid (EDTA) to chelate inhibitory metals.

Fig. 6 shows the inactivating effect of our papain under conditions of increasing proteolytic activity. The test phage was the tryp$^-$ mutant with added tryptophan. Curve 1 shows the effect on phage inactivation of papain in the non-proteolytic form : curve 2 in the presence of cysteine and curve 3 with cysteine and EDTA. The action of 1 on casein was negligible and of 3 very high.

It will be seen that curve 1 inactivated 90 per cent of phages in 30 min. at a dilution 1/500, while curve 2 has approximately the same effect at a dilution 1/5 and curve 3 only 35 per cent at 1/5. The higher concentrations of curve 1 invariably showed a smaller activity, but since this phenomenon has no relation to the action of tryptophan it will not be discussed in this place.

We may however conclude that the action of papain on phage is at a maximum when its proteolytic action is least, and conversely that high proteolytic action is associated with low action on phage.

There are 2 well known activities of papain, the protease and esterase both requiring activation by cysteine. Recently however a thiolesterase activity has been described (Johnston, 1956) and this can function without cysteine activation. It may therefore be said that the action on phage 3 is not due to protease, but may be due to the thiolesterase.

Inactivation of Phage by Antiphage Serum

An antiserum made in rabbits against phage tryp$^+$ was found to inactivate 50 per cent of the input in 1 hr. at 37° at a dilution varying between 1 in 18,000–27,000. It, however, was soon found that the titre was much higher on phage tryp$^+$ when free tryptophan was added; further, that the titre against phage tryp$^-$ was as high without added tryptophan.

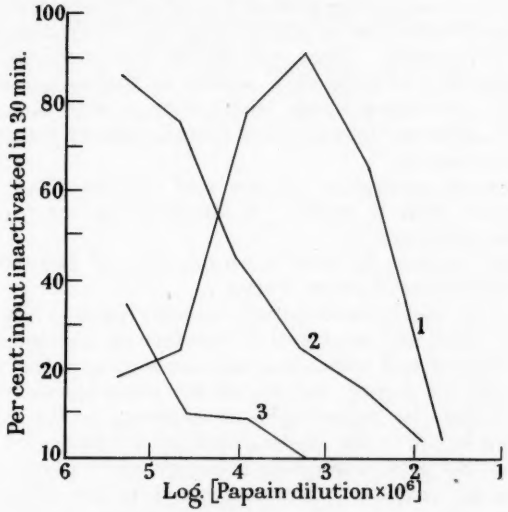


FIG. 6.—Effect of papain on Phage 3.
1. Papain alone.
2. Papain plus cysteine.
3. Papain plus cysteine and EDTA.

The effect of tryptophan in this case was not clear cut since there was marked inactivation without it even in dialysed serum. Efforts to separate a tryptophan-requiring component of the serum from another which operated without added tryptophan have not yet succeeded.

DISCUSSION

All these reactions which in the case of the tryp$^+$ phage require or are facilitated by tryptophan, result in irreversible inactivation of the phage. In the case of bacteria and minerals electron micrographs show that this is associated with adsorption by the tail, while the effect of heat is to cause destructive changes in the tail tip. For technical reasons satisfactory photographs of papain- or serum-inactivated phages have not been obtained.

The interest of these reactions is that they allow a reasonable conclusion that all have a common phase which is dependent on added tryptophan in a tryp$^+$ phage and a common site at the tail tip.

The nature of the reaction in which tryptophan participates is unknown, but at least it can be said that it is not a co-factor in adsorption since it accentuates the effect of mere heat. Neither can it be associated with some specific reaction between phage and bacterium because mineral particles can behave as bacteria. The recent work of Kozloff, Lute and Henderson (1957) and Kozloff and Lute (1957) was not immediately concerned with the action of tryptophan or adsorption of the tryptophan-requiring phage T4 to bacteria, but nevertheless opened up new possibilities.

These authors referred to the work of Johnston (1956) who found that papain contained a thiolesterase which at pH 7·5 breaks down synthetic thiolesters without activation by cysteine. They had shown that cyanide complexes of Zn group metals were able to split off a portion of the phage tail tip and in this process to rupture thiolester bonds which they conceived to be necessary to maintain the tail structure intact. The thiolesterase of papain also caused the same structural alterations.

Their references to adsorption are few, and of tryptophan, used with the T4 phage, they suggest that it causes " a distortion in the tail structure which exposes the susceptible bond ".

These proposals appear to have a bearing on our findings on the effect of tryptophan on adsorption of phage 3 tryp$^+$.

For instance, our results with papain strongly suggest that the effect is not due to protease action and may well be ascribed to the thiolesterase. In this case, following Kozloff and colleagues, we should accept the thiolester bond as the target not only for papain, but for all the other agents of inactivation and tryptophan could then be looked upon as exposing, in the tryp$^+$ phage, safely disposed thiolester bonds to the action of outside influences by " distorting " the tail or otherwise. So far as adsorption is concerned in a tryp$^+$ phage, the action of tryptophan is to " expose " thiolester bonds to the effect of a momentary adsorption on particulate matter. At temperatures above 0° this becomes irreversible owing to a release and spreading of some of the tail fibres. When tryptophan is absent in a tryp$^+$ phage, the bonds are not exposed and the presumed adsorption remains reversible without structural damage.

The tryp$^-$ mutant may be supposed either to have permanently a " distortion " of the tail without reference to tryptophan, or on the other hand it may have a permanent " distortion " induced by the invariable presence of tryptophan synthesised in the sites involved. Since the tryp$^-$ character is not entirely distinct from the tryp$^+$, each mutant carrying a proportion of the other and since in several of the reactions described in the tryp$^-$, the effect is increased by tryptophan, it is more likely that tryptophan is equally involved in the reactions of both phages but that in the tryp$^-$ it is structurally bound at the reacting sites.

SUMMARY

A number of reactions are described between a phage and other substances, which depend on the presence of tryptophan.

These reactions take place with a tryp$^-$ mutant without tryptophan.

Following the proposals of Kozloff and colleagues (1957) it is thought that the function of tryptophan is to cause exposure of thiolester bonds maintaining the structure of the phage tail. This leads to their subsequent rupture.

The tryp⁻ mutant is thought to have the thiolester bonds permanently exposed by virtue of tryptophan structurally attached.

REFERENCES

BOYD, J. S. K.—(1956) *Biol. Rev.*, **31**, 71.
CHENG, P.-Y.—(1956) *Biochim.-biophys. Acta*, **22**, 433.
FILDES, P. AND KAY, D.—(1957) *Brit. J. exp. Path.*, **38**, 563.
JOHNSTON, R. B.—(1956) *J. biol. Chem.*, **221**, 1037.
KALMANSON, G. M. AND BRONFENBRENNER, J.—(1943) *J. Immunol.*, **47**, 387.
KAY, D.—(1955) *Brit. J. exp. Path.*, **36**, 290.
KIMMEL, J. R. AND SMITH, E. L.—(1954) *J. biol. Chem.*, **207**, 515.
KOZLOFF, L. M. AND LUTE, M.—(1957) *Ibid.*, **228**, 529.
Iidem AND HENDERSON, K.—(1957) *Ibid.*, **228**, 511.
PUCK, T. T., GAREN, A. AND CLINE, J.—(1951) *J. exp. Med.*, **93**, 65.

HISTOCHEMICAL CHANGES IN RAT KIDNEY IN MAGNESIUM DEPRIVATION

R. HESS,* I. MACINTYRE, N. ALCOCK AND A. G. E. PEARSE

From the Postgraduate Medical School of London, Du Cane Road, London, W.12

Received for publication September 23, 1958

SINCE the original description by Kruse, Orent and McCollum (1932) of a characteristic syndrome of vasodilation and hyperexcitability, appearing in rats on a magnesium deficient diet, numerous reports have been published on chemical alterations in body fluids and tissues in magnesium deprivation (reviews by Greenberg, 1939 ; Elkinton, 1957 ; Hasselman and van Kampen, 1958). In experimental animals, organic lesions were found mainly in the kidney (Cramer, 1932 ; Watchhorn and McCance, 1937), myocardium (Greenberg, Anderson and Tufts, 1936) and cerebellum (Barron, Brown and Pearson, 1949). The changes in the kidney consisted of a progressive increase in the calcium content of that organ (Tufts and Greenberg, 1938) corresponding to the calcification of the inner cortical zone observed histologically. This lesion was found almost invariably in later phases of magnesium deficiency and was considered to precede (Greenberg, Lucia and Tufts, 1938) or to follow (Moore, Hallman and Sholl, 1938) more extensive morphological damage of tubular epithelium. Conventional histological techniques were unable to reveal any alteration in renal cells directly dependent on the induced electrolyte imbalance and leading to necrosis and calcification.

Magnesium ions are essential in the maintenance of mitochondrial structure and function (Raaflaub, 1953). It might be expected, therefore, that cellular changes from magnesium deprivation would most readily be made visible by histochemical methods capable of demonstrating mitochondrial alterations.

The present paper deals with the correlation, following dietary magnesium depletion, of biochemical data and histochemical alterations in the kidney as shown by the activities of various mitochondrial diaphorases and dehydrogenases. One other group of enzymes, the intracellular cathepsins, was studied by histochemical means alone.

MATERIALS AND METHODS

Young female rats (18) of the hooded strain (mean initial weight 95 g.) were placed on a magnesium deficient diet containing 0·25 mM Mg per kg. The diet of the control group of 10 rats contained 18 mM Mg per kg. In both groups the diet contained 150 mM Ca per kg. and was supplemented with vitamins and trace metals. Its exact composition is reported elsewhere (MacIntyre and Davidsson, 1958*b*).

The animals were killed at intervals from 3 to 50 days by exsanguination under nembutal anaesthesia. The right kidneys from 11 animals of the experimental group and from 3 animals of the control group were obtained for histochemical studies. In addition, psoas muscle was examined.

* Fellow of Swiss Foundation for Biological Medical Fellowships. Present address : Institute of Pathology, University of Basle, Switzerland.

For the demonstration of pyridine nucleotide-linked dehydrogenases and diaphorases in cryostat sections, the tetrazolium methods of Hess, Scarpelli and Pearse (1958*a* and *b*) and Scarpelli, Hess and Pearse (1958) were used. Succinic dehydrogenase was shown by the N-thiazolyl tetrazolium-cobalt chelation method of Pearse (1957*a*) modified by the addition of 7·5 per cent polyvinyl pyrrolidone and 10 mM amytal to the incubating medium. Mg^{++} was omitted from the substrate mixtures. Incubation was carried out at 37° for 15 min. in order to demonstrate the diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) diaphorases and succinic dehydrogenase, and for 30 min. to demonstrate the pyridine nucleotide-linked dehydrogenases.

Osmotic mitochondrial swelling was induced by pre-incubating freshly cut tissue sections in 0·06 M phosphate buffer pH 6·8, without substrate and co-enzyme, for 10 min. at 20°. Serial control sections were pre-incubated in buffered 0·25 M and 0·44 M sucrose. After this pretreatment the sections were transferred to the hypertonic succinic dehydrogenase medium mentioned above. Cathepsin-like activity in normal and damaged tubular cells was demonstrated in cold formalin-calcium fixed sections according to the method of Hess and Pearse (1958), except that in order to achieve a finer intracellular localization 4-chloro-5-bromo-*o*-acetyl indoxyl was employed in place of the 5-bromo derivative (Holt and Withers, 1958).

In addition to the enzyme methods, serial cryostat sections, fixed in 5 per cent acetic-ethanol, were stained for normal cytology by the Solochrome method (Pearse, 1957*b*), for lipids with Oil red O, and for calcium with 0·5 per cent aqueous naphthochrome green. Remaining parts of the removed kidneys were fixed in 10 per cent formol-calcium, embedded in paraffin, and 5 μ sections were stained with haematoxylin-eosin.

Chemical analysis.—Plasma magnesium, plasma calcium and kidney calcium were determined in all the experimental and control animals in solutions of plasma and fat-free tissue ash in N-HCl by flame photometry at wavelengths of 423 mμ and 285 mμ for Ca and Mg respectively. Full details of the methods used for analysis have been reported by MacIntyre and Davidsson (1958*b*).

RESULTS

Histochemical Methods

Mitochondrial structure.—In normal tissues, where the mitochondria have been protected against damage, the dehydrogenase and diaphorase activities are shown by the N-thiazolyl monotetrazolium-cobalt chelation method or by the Nitro-BT method, as intramitochondrial dots of 0·2–0·3 μ diameter. Each mitochondrion contains 2–5 dots separated by non-reactive areas (Pearse and Scarpelli, 1958). During the process of mitochondrial swelling, whatever its cause, the dots become progressively larger, corresponding to the increased size of the organelle (Scarpelli and Pearse, 1958).

Early changes in Mg^{++} deficiency.—With all the dehydrogenase and diaphorase methods used mitochondrial swelling in kidney tissue was already apparent after 3 days of Mg-deprivation (Fig. 1). This change corresponded to the initial fall in the level of plasma magnesium. Mitochondrial swelling was observed first in cells of the distal segment of the proximal convolution in the inner cortex (that segment terminating in the thick descending limb) and was restricted to a limited number of mitochondria per cell (Fig. 3). Most of the altered organelles were situated near the basement membrane. The swollen mitochondria, as demonstrated by the formazan deposits, were enlarged up to several microns in diameter, and they exhibited strong enzymic activity. In even more advanced stages lipid was shown to accumulate in the same tubules within the enlarged dots (Fig. 2), the enzymic activity now being restricted to a peripheral remnant (Fig. 3).

Pre-incubation of the fresh sections in 0·06 M buffer resulted in pronounced osmotic swelling of most of the mitochondria in the partially damaged areas (Fig. 4). Pre-incubation in isotonic (0·25 M) sucrose was followed by slightly

increased swelling of mitochondria in distal parts of proximal convolutions as compared to sections pre-incubated in hypertonic (0·44 M) sucrose. These findings suggest a lowered osmotic resistance of most of the organelles present in a cell that has undergone partial mitochondrial damage. In contrast, the normal appearing mitochondria in proximal convolutions of the outer cortex were not altered significantly by hypotonic isotonic pre-incubation. Addition of 5 mM adenosine triphosphate to the incubating medium had no effect on mitochondrial swelling.

At 6 *days*, as well as appearing in the inner cortex, mitochondrial swelling was observed in segmental parts of collecting ducts near the tip of the papilla (Fig. 5). There was also patchy mitochondrial swelling in cells of the medullary interstitial tissue and in the thin limb of Henle's loop.

At 9 *days*, necrosis of epithelia in limited segments of the distal part of the proximal convolutions was encountered. This lesion was the first to be recognizable in conventionally treated histological sections. The necrotic areas corresponded to sites where at the 3rd day of the experiment mitochondrial swelling was most pronounced. Intracellularly, fine calcium deposits were seen in some of the cells undergoing necrosis. Enzymatic activity was restricted to some mitochondrial remnants or was completely lost (Fig. 6). Some proximal convolutions in the outer cortex were distended. Here, as well as in some distal tubules a moderate swelling of mitochondria was observed. The change noted earlier in the collecting ducts had not progressed.

From 12 *to* 20 *days*, the mitochondrial swelling in the proximal convolutions of the outer cortex became more pronounced. Scattered areas of the middle parts of proximal tubules showed necrosis. Slight mitochondrial swelling was also

---

EXPLANATION OF PLATES

FIG. 1.—Reaction for DPN-diaphorase in the inner cortex shows mitochondrial swelling in terminal parts of a proximal convolution of a rat depleted for 3 days. × 900.

FIG. 2.—Same kidney as Fig. 1. Oil red O positive lipid droplets corresponding to the sites of most pronounced mitochondrial swelling. × 900.

FIG. 3.—Same tubular area as Fig. 1 at higher magnification. Marked swelling of some mitochondria as revealed by the succinic dehydrogenase reaction. Dark blue reactive sites (cobalt-formazan) are surrounded excentrically by a zone of faintly staining non-chelated red formazan, dissolved in lipid. × 3300.

FIG. 4.—Serial section to Fig. 3. Succinic dehydrogenase. Osmotic swelling of almost all the mitochondria after pre-incubation of the section in phosphate buffer for 10 minutes. × 3300.

FIG. 5.—Mitochondrial swelling and partial loss of enzymatic activity in segments of collecting ducts of the papilla. Reaction for glutamic dehydrogenase in an animal depleted for 6 days. × 900.

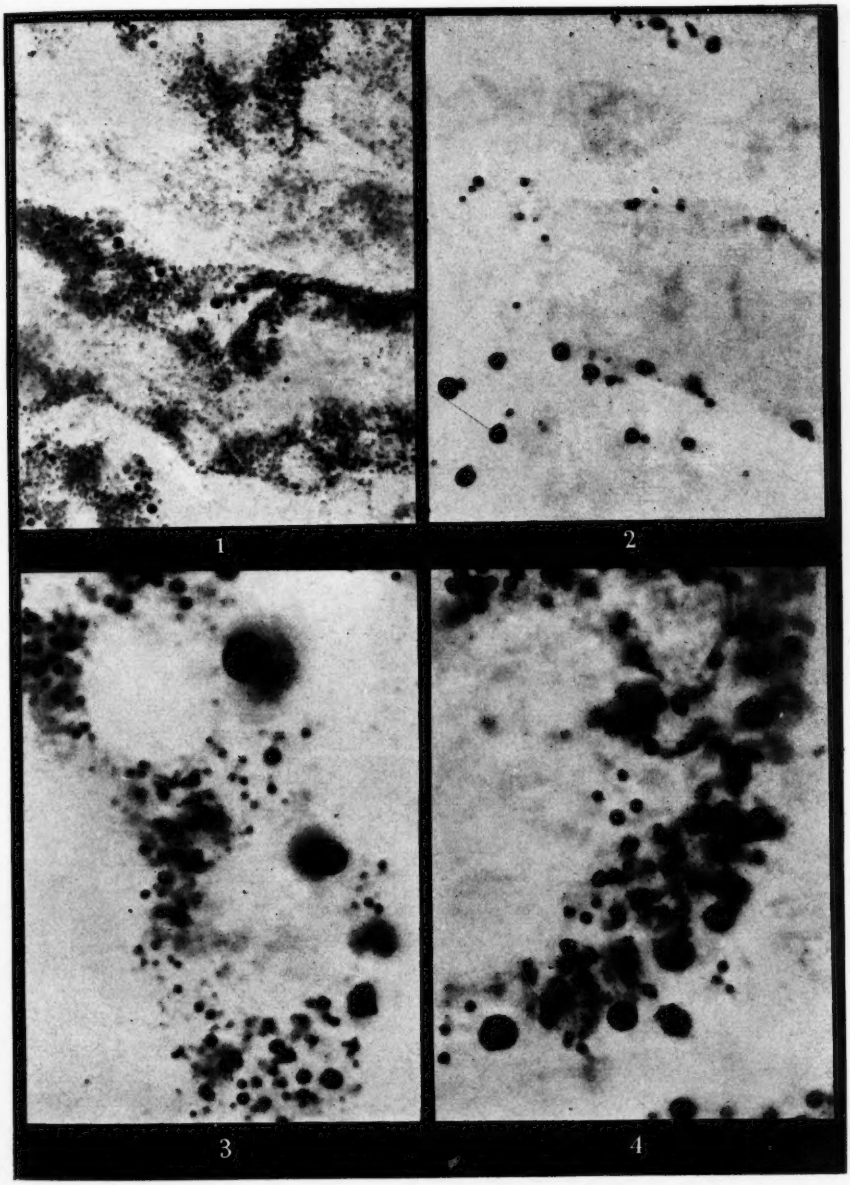
FIG. 6.—Marked decrease of TPN-linked isocitric dehydrogenase in terminal portions of a proximal convolution. Nine days' depletion. × 900.

FIG. 7.—After 12 days' depletion calcification of cellular debris is apparent in tubules of the inner cortex. Scattered small calcium deposits in cells undergoing necrosis. Naphthochrome green. × 900.

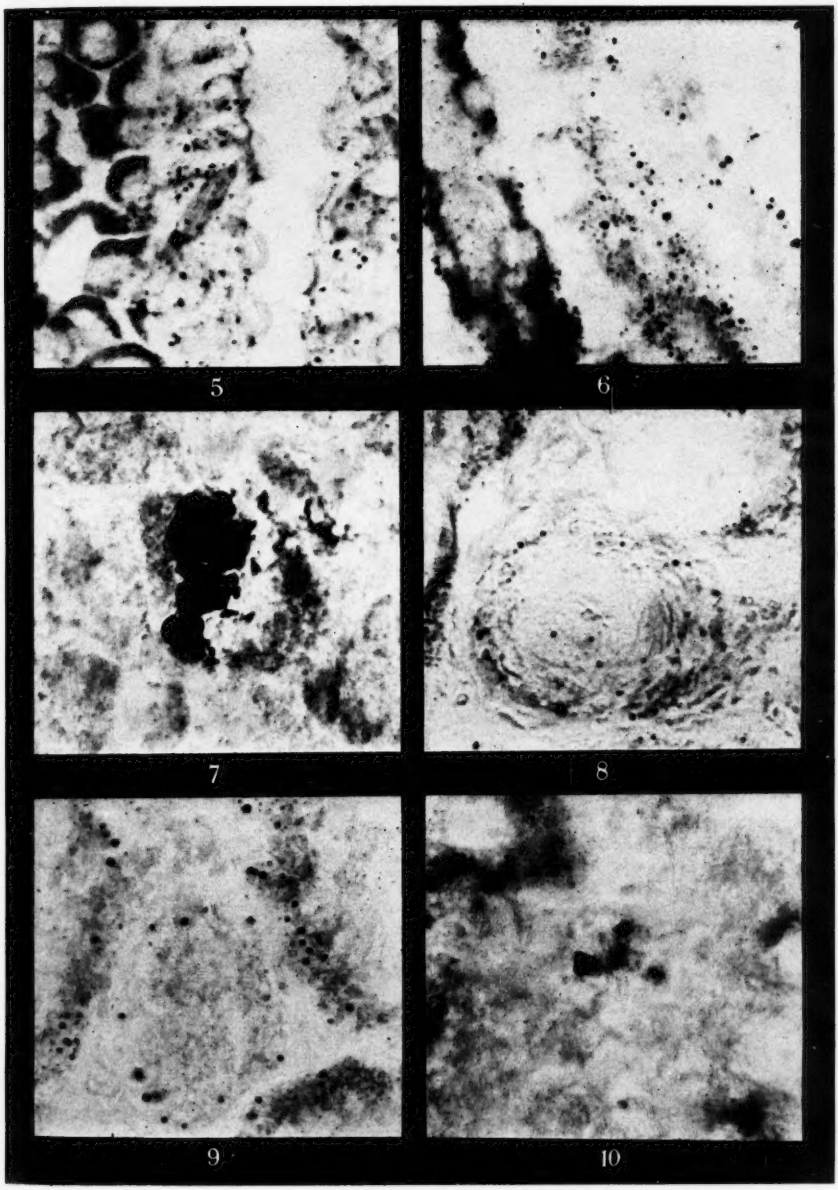
FIG. 8.—Almost complete loss of succinic dehydrogenase activity in a calcified area of the inner cortex. Fifteen days' depletion. × 900.

FIG. 9.—Mitochondrial swelling and decrease of succinic dehyrogenase in proximal convoluted tubules of the outer cortex. Fifty days' depletion. × 900.

FIG. 10.—Showing cathepsin-like activity of indoxylesterase in a desquamated tubular cell. Partially decreased activity in cells still attached to the basement membrane. Distal part of a proximal tubule, 9 days' depletion. × 2300.



Hess, MacIntyre, Alcock and Pearse.



Hess, MacIntyre, Alcock and Pearse.

noted in descending and ascending limbs of Henle's loop, whereas little alteration was seen in the papilla. The change observed earlier in mitochondria of the collecting duct apparently was partly reversible. Calcium deposits occurred throughout the inner cortex and occasionally were seen intracellularly in the outer cortex, confined to necrotic zones. In the distal part of the proximal tubules the deposits, which occurred first within the damaged cells, enlarged and became detached into the tubular lumen (Fig. 7), forming calcareous casts. The latter occluded the ducts and were surrounded by a thin cellular layer, mostly fibroblasts. Regeneration of tubular epithelium was seen infrequently.

From an animal kept for 14 days on the Mg-deficient diet, the mitochondrial fraction of the left kidney prepared in 0·44 M sucrose was obtained for microscopical study. The suspension was incubated in the DPN-diaphorase medium for 15 min. and mounted in 10 per cent gelatine. The mitochondria consisted of formazan bodies greatly varying in size, but closely corresponding to the different sizes of mitochondrial reactive sites in sections of the opposite kidney treated with the same incubating medium.

At 41, 48 *and* 50 *days*, very little further damage was seen in the kidneys except a progressive fibrosis in the inner cortex and the appearance of more calcareous deposits extending into the outer cortical zone. Here, mitochondrial swelling in proximal convolutions was slowly progressive (Fig. 9).

Psoas muscle exhibited sarcosomal swelling at the 20th day of the experiment.

Controls.—One animal out of 3 controls showed slight mitochondrial swelling in the outer cortex. There was no change in the inner cortex and medulla.

Enzyme reactions.—Mitochondrial swelling in its earlier phases was accompanied by a strong increase of diaphorase and dehydrogenase activities. More advanced swelling with lipid formation led to a loss of pyridine nucleotide-linked dehydrogenase activities, whereas succinic dehydrogenase still could be demonstrated. The damaged areas in the inner cortex suffered a very marked decrease in isocitric, malic, β-hydroxybutyric and lactic dehydrogenase activities. A reaction for α-glycerophosphate, alcohol and glutamic dehydrogenases could be obtained until the onset of necrosis. TPN-diaphorase and isocitric and malic dehydrogenases were increased in the collecting ducts throughout the experiment.

Calcification of cells was preceded (Fig. 6) and followed (Fig. 8) by an almost complete loss of enzymic activity.

Organophosphorus-resistant indoxyl esterase at pH 5·3 decreased in necrobiotic cells as long as they were still attached to the basement membrane. In contrast, necrotic cells desquamated into the lumen showed a strong esterase, corresponding to an increase in proteolytic activity (Fig. 10).

Chemical Analysis

The results obtained for plasma magnesium and plasma calcium from the whole group of experimental animals are given in Fig. 11. It is evident that after an initial fall in the first days of the magnesium privation, no further decrease of plasma Mg occurred. In fact, there was a slight tendency for an increase to subnormal values in later phases of the experiment. Plasma Ca rose after about 12 days. The table shows the value for kidney calcium and magnesium from a number of animals. The most marked change was the increase of kidney calcium. Kidney magnesium remained at normal values throughout the experiment.

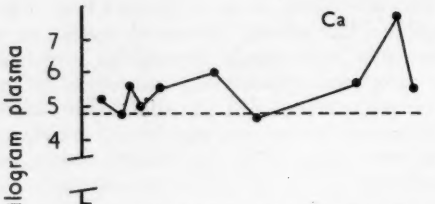


FIG. 11.—Plasma magnesium and plasma calcium in magnesium deficient rats, expressed in mEq per kg. plasma. The dotted lines show the mean values of magnesium (lower line) and calcium (upper line) from the control group.

TABLE.—*Kidney Calcium and Kidney Magnesium in Magnesium Deficient Rats*

Animal No.	Days on diet	Kidney Ca*	Kidney Mg*
Deficient†			
55	7	360	70
56	12	180	72
64	20	1630	88
62	50	1700	70
Controls‡			
85	51	24·3	76
91	58	21·6	73

* Expressed as mEq per kg. dry, fat-free tissue.
† 0·25 mM Mg per kg. diet.
‡ 18·0 mM Mg per kg. diet.

DISCUSSION

The results indicate that in growing rats, cytological changes occur in the kidney very early in magnesium deficiency. The intracellular alteration first observed consists of mitochondrial swelling and an increase of activity of oxidative enzymes in terminal segments of the proximal convoluted tubules. These changes are followed by destruction of mitochondria and loss of enzymic activity. The latter process, as observed after 9 days, was closely followed by cell necrosis and coincided with visible calcification in the same tubular area. Pronounced mitochondrial swelling was accompanied by the appearance of fat staining droplets in the tubular cells, but no other alteration could be detected in conventionally prepared histological sections, before the onset of necrosis.

The sequence of changes observed in the cytoplasm of tubular epithelia suggests that the kidney damage is the direct result of dietary magnesium deprivation and that it parallels the fall in plasma magnesium concentration which occurs in the first phase of the experiment.

It is interesting to note that the mitochondria within an altered cell vary in their response to magnesium deficiency. This observation suggests that not only do mitochondria isolated from different tissues show different sensitivity to agents capable of inducing swelling (Cooper and Tapley, 1957), but also that the mitochondria of a single cell respond differently to factors which influence their structure.

All the mitochondria of altered cells, as compared with normal cells, showed lowered osmotic resistance to a hypotonic medium.

The damage suffered by the distal portions of the proximal convoluted tubules, which results in calcification of the inner cortex, remains largely localized and is not followed by extensive alterations in other parts of the nephron. Proximal tubules of the outer cortex show only slowly progressive mitochondrial swelling and sensitivity to swelling agents and rarely necrosis. These findings may be related to the maintenance of total kidney magnesium during the course of magnesium deficiency. This is in contrast to the loss of magnesium from bone and muscle (MacIntyre and Davidsson, 1958*a*).

Since the mitochondrial changes observed were accompanied by a low plasma magnesium level it seems that in a normal animal the kidney cells depend on a constant magnesium supply. The protective effect of this ion on mitochondrial structure and function has been demonstrated *in vitro* by Raaflaub (1953). Ernster and Löw (1955) suggested that the integrity of isolated mitochondria and maintenance of oxidative phosphorylation are dependent on the presence of a magnesium-adenosine triphosphate complex which is dissociated by an increase in calcium ions. This transformation leads to mitochondrial swelling and loss of co-enzymes, with subsequent disruption of energy-producing mechanisms. The same type of *in vitro* alteration has been induced by Baltscheffsky (1957) by suspending mitochondria in a magnesium-free medium. It is conceivable that the mitochondrial changes seen in the kidney in the present experiments were an *in vivo* expression of the same phenomenon.

Whether the visible increase in intracellular calcium occurred as a response to a selective loss of magnesium from tubular cells, not detectable when the whole kidney is analysed, or by some other mechanism remains to be determined. An explanation might be that calcium is bound in the damaged cells by intermediates of the tricarboxylic acid cycle, especially citrate, which might possibly accumulate after a decrease in dehydrogenase activities.

Further, in view of the rapid development of the renal lesions associated with a low plasma magnesium and before an appreciable proportion of body magnesium is lost, hypomagnesaemia encountered clinically can no longer be dismissed as insignificant. In the authors' view the present results suggest that magnesium therapy may be indicated clinically whenever a low plasma magnesium persists.

SUMMARY

Using histochemical methods for the demonstration of various dehydrogenase and diaphorase activities, intracellular alterations in the rat kidney have been found to occur in the first days of dietary magnesium deficiency. This alteration was confined mainly to distal segments of the proximal convolutions and consisted of mitochondrial swelling closely followed by the appearance of lipid droplets. After 9 days, marked decrease of mitochondrial enzymic activities was found in

the damaged areas initiating intracellular calcification and necrosis. A close relationship was shown to exist between a decrease of plasma magnesium, an increase of plasma calcium and the mitochondrial alteration observed. Visible renal calcification was accompanied by a marked rise of kidney calcium content. No change of renal magnesium content was found throughout the experiment.

The present histochemical findings in magnesium deficient animals suggest that mitochondria differ markedly in their response to injury in the various segments of the nephron.

This work was facilitated by a Grant in Aid to one of us (A. G. E. P.) from the Medical Research Council and by a Grant from the Central Council for the Care of Cripples for the development of the cold microtome (cryostat).

We have to thank Dr. E. Kambli for the gift of a sample of the substituted indoxyl acetate, Mr. W. Brackenbury for the photomicrographs and Mr. F. Saunders for the text figure.

REFERENCES

Baltscheffsky, H.—(1957) *Biochim. biophys. Acta*, **25**, 382.
Barron, G. P., Brown, S. O. and Pearson, P. B.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **70**, 220.
Cooper, C. and Tapley, D. F.—(1957) *Biochim. biophys. Acta*, **25**, 426.
Cramer, W.—(1932) *Lancet*, ii, 174.
Elkinton, J. R.—(1957) *Clin. Chem.*, **3**, suppl. 319.
Ernster, L. and Löw, H.—(1955) *Exp. Cell Res.*, suppl. **3**, 133.
Greenberg, D. M.—(1939) *Ann. Rev. Biochem.*, **7**, 269.
Idem, Anderson, C. E. and Tufts, E. V.—(1936) *J. biol. Chem.*, **114**, xliii.
Idem, Lucia, S. P. and Tufts, E. V.—(1938) *Amer. J. Physiol.*, **121**, 424.
Hasselman, J. J. F. and van Kampen, E. J.—(1958) *Clin. Chim. acta*, **3**, 305.
Hess, R. and Pearse, A. G. E.—(1958) *Brit. J. exp. Path.*, **39**, 292.
Idem, Scarpelli, D. G. and Pearse, A. G. E.—(1958*a*) *Nature*, **181**, 1531.—(1958*b*) *J. biophys. biochim. Cytol.* (in press).
Holt, S. J. and Withers, R. F. J.—(1958) *Proc. Roy. Soc.*, B, **148**, 520.
Kruse, H. D., Orent, E. R. and McCollum, E. V.—(1932) *J. biol. Chem.*, **96**, 519.
MacIntyre, I. and Davidsson, D.—(1958*a*) *Biochem. J.*, **69**, 6P.—(1958*b*) *Ibid.* (in press).
Moore, L. A., Hallman, E. T. and Sholl, L. B.—(1938) *Arch. Path.*, **26**, 820.
Pearse, A. G. E.—(1957*a*) *J. Histochem. Cytochem.*, **5**, 515.—(1957*b*) *Acta Histochem.*, **4**, 95.
Idem and Scarpelli, D. G.—(1958) *Nature*, **181**, 702.
Raaflaub, J.—(1953) *Helv. Physiol. Pharmacol Acta*, **11**, 142.
Scarpelli, D. G. and Pearse, A. G. E.—(1958) *J. Histochem. Cytochem.* **6**, 326.
Idem, Hess, R. and Pearse, A. G. E.—(1958) *J. biophys. biochem. Cytol.* (in press).
Tufts, E. V. and Greenberg, D. M.—(1938) *J. biol. Chem.*, **122**, 693.
Watchhorn, E. and McCance, R. A.—(1937) *Biochem. J.*, **31**, 1379.

POLIOMYELITIS VIRUS FLOCCULATION: THE REACTIVITY OF UNCONCENTRATED CELL-CULTURE FLUIDS

GILLIAN M. CHURCHER,* F. W. SHEFFIELD AND WILSON SMITH

From the Department of Bacteriology, University College Hospital Medical School, London, W.C.1

Received for publication October 2, 1958

A DIRECT flocculation reaction with poliomyelitis viruses of the 3 classical types and antisera obtained by immunization of rabbits was described by Smith, Sheffield, Lee and Churcher (1956). The reaction was shown to be strictly type-specific and to occur also with some human sera obtained from cases of poliomyelitis at early stages of the infection. It was suggested therefore that it might eventually provide a simple and rapid method of diagnosis and virus type differentiation, but that essential preliminary work must include the production of large quantities of the virus antigens of standardised potency, followed by systematic investigation of a large number of human sera of diverse provenance. A major obstacle to progress was the apparent necessity of concentrating infective culture fluids by high-speed centrifugation, giving small yields of antigens from relatively large volumes of culture; for the unconcentrated culture fluids, with infectivity titres then considered to be the maximum attainable, were quite devoid of flocculating activity. The difficulty was partly met by the development of a micro method of flocculation requiring only 0·02 ml. of antigen per tube (Smith *et al.*, 1956). This method has proved to be invaluable in subsequent work but is less suitable than the Dreyer tube agglutination technique for routine diagnostic tests; it is less accurate, more laborious and more dependent on subjective factors. Moreover the unpredictable instability of the finely particulate concentrated antigens introduced further problems which have not yet been solved and high-speed centrifugation of cultures of virulent poliomyelitis virus strains entails some risk of laboratory infections.

The present paper reports the use of unconcentrated virus culture fluids as flocculating antigens. The results are still of a preliminary nature, but they do indicate the possibility of obtaining antigens in quantities adequate for routine use with reduced risks of accidental infections. They thus represent a significant advance towards the ultimate objective in view.

MATERIALS AND METHODS

Poliomyelitis virus strains

Three virulent strains, Brunhilde, Y–SK and Leon, and 3 attenuated virus strains were used. The attenuated viruses were the Mahoney L–Sc 2ab, P 712 Ch 2ab and Leon 12ab strains of Sabin (1957). A seed pool of each virulent strain was prepared from cultures using a cell line Hu-Li derived from human embryo liver; these were stored at − 20°. Seed pools of the attenuated viruses were prepared from cultures using ERK 1 cells derived from rabbit embryo kidney and stored at 4°. Both these lines of " transformed " cells were isolated and characterised by Westwood, Macpherson and Titmuss (1957).

* Supported by the National Fund for Poliomyelitis Research.

ERK 1 cell propagation

Two sub-lines of the ERK 1 cells were used.

Line ERK 1(A) was propagated serially in medium containing rabbit serum and was used for all virus infectivity titrations by the method described by Sheffield and Churcher (1957).

Line ERK 1(D) was used for the preparation of the flocculating antigens. It was grown in a medium consisting of bovine serum 10 per cent, tryptic digest broth 5 per cent, 5 per cent solution of lactalbumin hydrolysate 5 per cent and Earle's solution containing 0·11 per cent concentration of sodium bicarbonate 80 per cent. Penicillin 100 units per ml. and streptomycin 100 μg. per ml. were incorporated. Serial propagation was effected by trypsinisation of confluent cell cultures, resuspension of the cells in fresh medium to a cell concentration of 30,000 per ml. and distribution into either Pyrex babies' feeding bottles (10 ml. per bottle) or Roux bottles (100 ml. per bottle). Bottles were incubated in a stationary position at 36°; the medium was changed on the 4th day and confluent cell mono-layers were obtained by the 7th day. The confluent cultures in the babies' bottles contained approximately 4×10^6 cells, those in Roux bottles approximately 40×10^6 cells.

Preparation of flocculating antigens

A two-stage method was used. For virulent strain antigens cultures of ERK 1 (D) cells grown to confluence on one side of a baby's bottle were selected. The medium was removed and the cells washed twice with Earle's solution. Medium 199, 10 ml. and virus seed, 0·1 ml. were added to each bottle. After incubation on a roller machine for 72 hr. the cultures were harvested, pooled and centrifuged at 1700 r.p.m./10 min. to remove cell debris. The fluid was then held at room temperature for several days in a lightly plugged vessel to allow escape of CO_2 with consequent increase of alkalinity. It was then used to initiate the second stage of virus production.

For the second stage ERK 1 (D) cells distributed in confluent sheets over all 6 glass faces of babies' feeding bottles were used. This was achieved by seeding each bottle with the total cell harvest from a Roux bottle confluent culture (approximately 40×10^6 cells), suspended in 50 ml. medium and placing it immediately on a roller machine at 36°. On the 2nd day the medium was removed and replaced by 4·4 ml. first-stage virus fluid plus 0·5 ml. normal rabbit serum and 0·1 ml. 5 per cent glucose solution. After 2 or 3 days' further incubation on the roller machine fluids were harvested, pooled and centrifuged. The product was a very high-titre virus fluid suitable for use as antigen in micro-flocculation tests, but too turbid for Dreyer tube tests. It was therefore clarified by filtration through Seitz FCB or GS pads which had been thoroughly washed with saline and nutrient broth.

For the attenuated strain antigens slight modifications were needed. Medium CSV 8 (Cooper, Wilson and Burt, personal communication) was substituted for Medium 199 and the primary stage virus fluid was neutralised with N/10 HCl. The inoculum per bottle for the second stage was 4·1 ml. first-stage fluid, 0·5 ml. rabbit serum, 0·1 ml. 5 per cent glucose solution and 0·3 ml. 2·75 per cent solution of sodium carbonate.

Flocculation techniques

Both the macroscopic tube and the micro methods were used. These have been fully described in our previous publications. Dilutions of both antigens and antisera were expressed as final dilutions.

Flocculating antisera

These were obtained by the intravenous inoculation of rabbits with centrifuge-concentrates of virus as described by Smith *et al.* (1956). When the sera from the primary courses of immunization were exhausted the rabbits were re-immunized with booster doses. The use of sera from different bleeds having different antibody titres therefore introduced a variable factor but this does not invalidate the conclusions to be drawn from the experimental results.

EXPERIMENTAL

Flocculation of Virulent Strain Culture Fluids

The first successful flocculation of an unconcentrated culture fluid was obtained with Brunhilde virus. The antigen was obtained by the two-stage growth tech-

nique described above. It was tested by a chessboard titration against homologous rabbit antiserum using the macroscopic tube method. Serial two-fold dilutions of antigen and antiserum were made in saline and mixtures containing 0·3 ml. volumes of each reagent were made in Dreyer agglutination tubes. These were incubated at 37° in a water bath with only the bottom third of the fluid columns immersed. Observations at frequent intervals showed that flocculation was progressive up to 6 hr. Y-SK and Leon antigens were then prepared and tested in a full cross titration.

The results of both experiments at 6 hr. incubation are recorded in Table I. They show that strictly type specific flocculation occurred with each culture fluid antigen. The antigens differed in flocculation potency which to some extent was probably a reflection of their different infectivity titres. Infectivity titres were $10^{8.9}$, $10^{8.0}$ and $10^{8.7}$ TCD_{50} per ml. for Brunhilde, Y-SK and Leon respectively. The antigen and antibody end points, however, were of the same order as those obtained previously with centrifuge-concentrated antigens and products with this degree of flocculating potency would certainly be adequate for routine use. Although the floccules are smaller than those obtained with concentrated particulate suspensions, the complete clarity of the filtered culture fluids greatly facilitates detection of " trace " reactions and difficulties arising from instability of concentrated antigens are eliminated.

Flocculation of Attenuated Strain Culture Fluids

No strictly homologous antisera were available. Culture fluids of each virus type were therefore tested against the rabbit antisera prepared against the virulent virus strains. Full chessboard cross titrations were done by the macroscopic tube method. The results, recorded in Table II show that typical type specific flocculation patterns were obtained and, unexpectedly, the antigen titres were approximately equal to those of the virulent strain fluids. Higher titres would certainly be advantageous and it is reasonable to expect that they will be attainable by further modifications of the conditions for virus growth.

Comparative Tests of Two-stage and Suspended Cell Culture Antigens by the Macroscopic and Microscopic Flocculation Techniques

Whilst all the antigens hitherto prepared by the two-stage method have had satisfactory flocculation potency, culture fluids from single-stage virus growth have mostly been completely inactive. During the progress of the work, however, we were informed that unconcentrated virus fluids derived from suspended ERK cell cultures had been found to give flooculation with our rabbit antisera (Appleyard and Westwood, unpublished) and several batches of such antigens were sent to us by these workers for test. We were also supplied with various batches of attenuated strain cultures by Cooper and his associates of the M.R.C. Virus Culture Laboratory, Carshalton.

Conservation of our potent rabbit antisera demanded preliminary screening of these potential antigens with the greatest possible economy in use of reagents. The preliminary screening of small samples of human sera is also likely to be required in future. In spite of the advantages of the Dreyer tube macroscopic flocculation method, enumerated above, it is too extravagant for this purpose. A one-dimensional test by the micro method was considered to be ideal but it was

TABLE I.—*Flocculation of Unconcentrated Culture Fluids of Virulent Strains*

Antigen	Antigen dilu.	Antisera: Brunhilde: 1 in: 20	40	80	160	320	640	1280	Antisera: Y-SK: 1 in: 20	40	80	160	320	640	Antisera: Leon: 1 in: 20	40	80	160	320	640	Saline control
Brunhilde	.	+	+	+	+	+	+	−	−	. .	. .	. .	. .	. .	. .	−	. .	. .	. .	. .	−
	½	+	+	+	+	+	+	+	−	. .	. .	. .	. .	. .	. .	−	. .	. .	. .	. .	−
	¼	+	+	+	+	+	+	+	−	. .	. .	. .	. .	. .	. .	−	. .	. .	. .	. .	−
	⅛	+	+	+	+	+	+	+	−	. .	. .	. .	. .	. .	. .	−	. .	. .	. .	. .	−
	1/16	.	+	−	−	−	+	+	−	. .	. .	. .	. .	. .	. .	−	. .	. .	. .	. .	−
Y-SK	.	.	.	.	.	.	.	.	+	+	+	+	+	+	−	. .	. .	. .	. .	. .	−
	½	.	.	.	.	.	.	.	+	+	+	+	+	+	−	−	. .	. .	. .	. .	−
	¼	.	.	.	.	.	.	.	+	+	−	+	+	−	−	−	. .	. .	. .	. .	−
	⅛	.	.	.	.	.	.	.	−	+	−	−	−	−	−	−	. .	. .	. .	. .	−
Leon	.	.	.	.	.	.	.	.	−	−	. .	. .	. .	. .	+	+	+	+	+	+	−
	½	.	.	.	.	.	.	.	−	−	. .	. .	. .	. .	+	+	+	+	+	−	−
	¼	.	.	.	.	.	.	.	−	−	. .	. .	. .	. .	+	+	+	+	+	−	−
	⅛	.	.	.	.	.	.	.	−	−	. .	. .	. .	. .	+	−	+	+	+	−	−
Saline controls	. .	−	. .	. .	. .	. .	. .	. .	−	−	. .	. .	. .	. .	−	−	. .	. .	. .	. .	. .

In all tables degree of flocculation is indicated by number of + signs; op indicates opalescence without detectable particulation; − indicates no detectable reaction.

TABLE II.—*Flocculation of Unconcentrated Culture Fluids of Attenuated Strains*

Antigen	Antigen dilu.	Antisera: Brunhilde: 1 in: 40	80	160	320	640	1280	2560	Antisera: Y-SK: 1 in: 40	80	160	320	640	1280	Antisera: Leon: 1 in: 40	80	160	320	640	Saline
Mahoney L-SC 2ab	.	+	+	+	+	+	−	−	+	−	−	+	+	+	. .	−	. .	. .	. .	−
	½	+	+	+	+	+	+	−	+	+	−	+	+	+	. .	−	. .	. .	. .	−
	¼	+	+	+	+	+	+	−	+	+	−	−	−	−	. .	−	. .	. .	. .	−
	⅛	.	+	+	+	+	+	−	+	+	−	−	−	−	. .	−	. .	. .	. .	−
	1/16	.	.	−	−	−	−	−	−	−	−	−	−	−	. .	−	. .	. .	. .	−
P712 Ch 2ab	.	.	−	. .	. .	. .	. .	. .	+	+	−	+	+	+	. .	−	. .	. .	. .	−
	½	.	−	. .	. .	. .	. .	. .	+	+	−	+	+	+	. .	−	. .	. .	. .	−
	¼	.	−	. .	. .	. .	. .	. .	+	+	−	−	−	−	. .	−	. .	. .	. .	−
	⅛	.	−	. .	. .	. .	. .	. .	−	−	−	−	−	−	. .	−	. .	. .	. .	−
	1/16	.	.	. .	. .	. .	. .	. .	.	−	−	−	−	−	. .	−	. .	. .	. .	−
Leon 12ab	.	.	−	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	++	+	+	−	−
	½	.	−	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	++	+	+	−	−
	¼	.	−	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	++	+	+	−	−
	⅛	.	−	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	++	+	−	−	−
	1/16	.	.	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	+	−	−	−	−
Saline	.	.	.	. .	. .	. .	. .	. .	.	−	−	. .	. .	. .	. .	−	. .	. .	. .	.

essential first to establish its validity by a direct comparison of the two methods. The experiment was designed to compare also the flocculation behaviour of virus fluids obtained by the two-stage and by the suspended cell culture techniques.

Two type 1 Brunhilde antigens were used, one freshly prepared by the two-stage culture method, the other prepared by Appleyard and Westwood from suspended cell cultures of ERK cells. They were each tested in full chessboard titrations by both macro and micro flocculation methods. The tests were done on the same day with serum dilutions made in bulk to eliminate possible misinterpretation from errors of dilution.

Progressive development of the reaction in macroscopic tube flocculation was observed by readings at 1, 4, 6 and 24 hr. incubation. This unfortunately is not possible in the micro method because of the time involved in the serial examination of hanging drop preparations by dark ground microscopy. A single reading of the micro tests was therefore made after 4 hr. incubation. The results are recorded in Table III, but the 24 hr. readings are omitted because by this time the reaction in every tube showing any degree of flocculation at 6 hr. had progressed to total flocculation, but without any appreciable extension of the flocculation " area " as a whole.

The superiority of the two-stage culture antigen is clearly apparent. It gave more rapid development and extension of flocculation, larger aggregates in corresponding serum dilution tubes and larger flocculation areas with reactions at higher dilutions of both antigen and antiserum. This is in line with the respective infectivity titres of the two fluids which were $10^{8.5}$ and $10^{7.8}$ TCD_{50} per ml. It should be noted, however, that the antigens supplied by Appleyard and Westwood were the earliest batches to be produced by their suspended cell culture technique so that this relatively lower potency does not necessarily indicate any intrinsic inferiority of the method. On the contrary, it is reasonably certain that, with modifications, suspended cell cultures will eventually yield antigens fully equal to those obtained by the two-stage virus growth method with the advantage of much greater flexibility for large scale production. The 4 hr. readings of macro and micro tests show closely similar reaction patterns, but with an indication that the micro test may be slightly more sensitive. Any advantage from such increased sensitivity is offset by the difficulty of detecting trace reactions by microscopic observation of single drop samples. The validity of the micro method, however, for screening tests is fully established.

Flocculability of Formalinised Culture Fluid Antigens

In previous work with centrifuge-concentrated antigens little trouble was encountered from bacterial contamination, probably because of lack of nutrient materials in the saline virus suspensions. On the other hand the unconcentrated culture fluids, containing cell breakdown products in a serum enriched medium, promote luxuriant growth of any bacterial contaminants, entry of which is difficult to prevent over long periods of use as flocculating antigens. The effect of formalin on flocculation potency was therefore investigated with a view to its incorporation as a bacteriostatic agent. Formalin was chosen because its employment in poliomyelitis virus vaccine production pointed to its possible future use in the development of flocculating antigens completely free from living infective virus.

A type 1 Brunhilde virus fluid obtained from monolayer ERK cell cultures was used in preference to a more potent two-stage virus growth antigen. To

TABLE III.—*Comparison of Tube and Micro Flocculation Techniques Using Brunhilde Tissue Culture Fluid Antigens Prepared by Two Different Methods*

Technique flocculation	Time (hr.)	Antigen dil.	Monolayer antigen (2-stage method)											Suspended cell antigen										
			Brunhilde antiserum : 1 in :							Y-8K	Leon	Saline		Brunhilde antiserum : 1 in :							Y-8K	Leon	Saline	
			15	30	60	120	240	480	960	1920	60	60		15	30	60	120	240	480	960	1920	60	60	
Tube	1													op	op	op						op		
														op	op	op								
														op	op									
	4													op	op									
														op	op									
														op	op									
	6													op										
														op										
														op										
		Saline																						
Micro method	1																							
	4																							
		Saline																						

aliquots of the fluid one-ninth volumes of saline, 1 in 400 dilution formalin, and 1 in 200 dilution formalin, were added. The mixtures were kept at 4° and tested at intervals without prior neutralisation of residual formalin. Line tests by the micro method were employed. The results summarised in Table IV show that the incorporation of formalin in a concentration of 1 in 2000 is not detrimental to the use of culture fluids as flocculating antigens over a storage period of more than 3 months. Formalinised fluids kept longer than this have not yet been tested. In this experiment no infectivity tests were done; the production of antigens completely devoid of living virus by means of formalin and other virus-inactivating agents will be reported in a subsequent publication.

TABLE IV.—*Flocculation Reaction with Formalinised Virus-culture Fluids*

Storage 4° (days)	Culture-fluid antigen (Brun.)	Brun. antiserum : 1 in. : 20	40	80	160	320	640	Y-SK serum 20	Leon serum 20	Ant. sal. control
10	Untreated	+	+	+	±	−	..	..	..	−
	Form. 1/4000	++	+	+	±	−	..	..	..	−
	,, 1/2000	++	+	+	±	−	..	..	..	−
25	Untreated	++	+	+	+	±	..	−	−	−
	Form. 1/4000	++	+	+	+	±	..	−	−	−
	,, 1/2000	++	+	+	+	±	..	−	−	−
107	Untreated	++	++	+	+	±	−	−	±	−
	Form. 1/4000	++	++	+	+	−	−	−	±	−
	,, 1/2000	++	++	+	+	±	−	−	±	−

Heterotypic Cross Reactions

Table IV shows that in the test at 107 days, some degree of flocculation occurred in the control mixtures containing the Brunhilde antigens and Leon antiserum. The reason for this is that the Leon antiserum used was a sample obtained after re-immunisation of the rabbit with booster doses of ERK cell culture virus. Tests of serum samples from serial bleeds gave heterotypic cross reactions with all samples after re-immunisation, but not with earlier samples. Tests of Y-SK sera from serial bleeds gave a similar result. These cross reactions are not due to heterotypic poliomyelitis virus antibodies for they are abolished by absorption of the sera with ERK cells without reduction of the type specific flocculation titre (Appleyard and Westwood, personal communication). They indicate that in spite of their rabbit origin ERK cells contain a component which is immunogenic for the rabbit. The implications of this phenomenon are discussed below.

DISCUSSION

The development of techniques for producing poliomyelitis virus culture fluids, which will flocculate specifically with homologous antisera without the necessity of virus concentration or purification, should greatly increase the potentialities of the reaction. Systematic investigation of the dynamics of the reaction will be facilitated by the use of the macroscopic tube test in which continuous observation is possible but this requires considerable quantities of antigens which have been freed from visible particles. The preliminary work

reported shows it is possible to produce these for each virus type with both virulent and attenuated strains. Moreover their production by different methods of virus culture suggests that much more potent antigens will eventually result from further technical developments.

Previous failure to obtain flocculation with unconcentrated culture fluids was almost certainly due mainly to their relatively low content of virus particles, as reflected by their infectivity titres. It is doubtful, however, whether this is the sole determinant factor. Attempts to obtain flocculating antigens from the virus fluids of monkey kidney culture have been unsuccessful, even with centrifuge-concentration to offset their lower initial virus concentrations. There is some evidence that poliomyelitis virus flocculation may be prevented by the presence of non-specific inhibitors so that the type of cell used for virus propagation may be crucial. It may be significant that all the flocculating antigens produced as yet, either with or without centrifuge-concentration, have been derived either from HeLa cells or from one of the transformed cell lines of Westwood, and there is now little doubt that HeLa cells have also undergone the phenomenon of transformation. It is true that gel-diffusion precipitation is given by virus grown in monkey kidney cells, but diffusion may be an effective way of separating virus from inhibitor substances.

HeLa cells were originally used for production of our flocculating antigens. They were replaced by ERK cells for several reasons, as soon as these were found to be susceptible to infection with poliomyelitis viruses. ERK cells are more easily maintained in serial passages and grow well in bovine or rabbit serum-containing media. They are less liable to spontaneous degenerative changes and are thus superior for virus infectivity titrations. They yield higher titre virus fluids under comparable conditions (Sheffield and Churcher, 1957). The chief reason for their adoption, however, was because it was thought that their rabbit origin would obviate the elicitation of antibodies against any cell material which might remain in virus suspensions used for rabbit immunisation. In view of recent studies by several independent workers it is not now surprising that such antibodies do appear in the course of rabbit hyperimmunization. The phenomenon of cell transformation appears to be associated with a change of antigenic structure reflected by loss of the original species markers and acquisition, or unmasking, of an antigen common to transformed cells whatever their species origin (Westwood *et al.*, 1957 ; Melnick and Habel, 1958). This must be taken into account in any use of antisera against viruses grown in cells of this type. It is not likely to affect tests of human sera from cases of poliomyelitis but may conceivably do so with post-vaccination sera. Fortunately removal of the cross reacting antibodies by absorption with cells presents no difficulty.

The exceptionally high virus infectivity titres attained by the two-stage cultivation technique and the successful use of suspended ERK cells for production of flocculating antigen are cogent arguments for the adoption of these cells for virus vaccine production, if and when the hypothetical risks associated with potential malignancy are judged to be negligible.

SUMMARY

A two-stage method of poliomyelitis virus cultivation in ERK cells is described which yields virus fluids of exceptionally high infectivity titres.

The virus fluids are effective as flocculating antigens without prior virus concentration. They can be freed from visible particles and rendered bacteriologically sterile by Seitz filtration without loss of potency.

Effective flocculation antigens can be obtained with either virulent or attenuated strains of the 3 virus types.

Preliminary experiments indicate that formalinised antigens stored at 4° retain flocculation potency for over 3 months.

The work was aided by a grant from the National Foundation (U.S.A.). Expenses were also partly defrayed by grants from the National Fund for Poliomyelitis Research and the Medical Research Council.

We are greatly indebted to Dr. J. C. N. Westwood and Dr. G. Appleyard of M.R.E., Porton, for the virus fluids prepared in suspended ERK cell cultures, and to Dr. P. D. Cooper of the M.R.C. Virus Culture Laboratory, Carshalton, for seed viruses of the attenuated strains.

REFERENCES

MELNICK, J. L. AND HABEL, K.—(1958) *Fed. Proc.*, **17**, 526.
SABIN, A. B.—(1957) *Special Publications, New York Academy of Sciences*, **5**, 113.
SHEFFIELD, F. W. AND CHURCHER, G. M.—(1957) *Brit. J. exp. Path.*, **38**, 155.
SMITH, W., SHEFFIELD, F. W., LEE, L. M. AND CHURCHER, G. M.—(1956) *Lancet*, i, 710.
Idem, SHEFFIELD, F. W., CHURCHER, G. M. AND LEE, L. M.—(1956) *Ibid.*, ii, 163.
WESTWOOD, J. C. N., MACPHERSON, I. A. AND TITMUSS, D. M. J.—(1957) *Brit. J. exp. Path.*, **38**, 138.

SURVEY OF PAPERS

LAUFER, CHLOE TAL and BEHAR have examined the effects of injected Freund's adjuvent upon mice, guinea-pigs and Syrian hamsters. They found that proliferative inflammatory lesions in the brain occurred only in the hamsters. All 3 species of animal showed reactions of similar nature in the liver, spleen and lungs (p. 1).

LAWRENCE has studied the effects of Staphylococcal and Streptococcal toxins upon skin respiration and the migration and viability of epithelial cells in tissue culture. He found Staphylococcal α toxin to be extremely toxic and rapid in its action. Streptolysin 0 was rather less toxic than staphylococcal α toxin, but also rapid in its action. Streptolysin S was relatively non-toxic to the cultures. Staphylococcal anti-toxin, if added more than an hour after α toxin, was unable to prevent the toxic effects of the toxin upon the skin (p. 8).

HOBSON, PORTER and WHITBY administered bone marrow from rabbits previously immunized with bovine γ-globulin to rabbits that had received massive whole body X-irradiation: the recipients were challenged with BGG^{131}I 1 hour or 10 days after receiving marrow. At 1 hr. the antigen was eliminated as in immune animals. At 10 days no increase in the rate of removal was demonstrated. Survivors received a second dose of BGG after 5 weeks and those with successful transplants acted as immune animals (p. 15).

KRISHNA MURTHY investigated the vascular pattern of primary tumours in the livers of rats induced by the administration of " Butter Yellow ". Indian ink was perfused into the portal circulation under ether anaesthesia and the livers removed. The investigations were conducted at time intervals from 30–360 days and the changing vascular patterns described. It was found that the liver tumours depended on the hepatic artery for their nutrition and not on the portal vein (p. 25).

ROBSON and SMITH injected *Myco. lepraemurium* into the cornea of mice and studied the course of the resulting local lesion. They have developed a technique of counting the number of mycobacteria in an individual cornea based upon digestion of the cornea by an enzyme mixture (p. 33).

POWELL and GOUGH gave powdered quartz intratracheally to rabbits which later were rendered hypersensitive by injection of horse serum intravenously. The resulting silicotic lesions were larger, more clearly demarcated, contained more collagen and more aggregated dust in the hypersensitive animals than in the controls (p. 40).

SCHWARTZ evaluated the role of the ground substance changes of lathyrism in experimentally produced atherosclerosis. Atheroma was induced in rabbits by cholesterol feeding and during the experiment the test group received aminopropionate (BAPN) for a period of 56 days. Serum levels of cholesterol, phospholipid, hexosamine and mucoprotein were estimated. At necropsy the degree of atheroma was recorded and histochemical investigations of the material were performed.

The skeletal and aortic lesions of lathyrism were produced accompanied by significant increases in the serum levels of hexosamine and mucoprotein. Foam cell deposition was produced at the sites of lathyrus lesions and the possible role of mucopolysaccharide as the tissue factor controlling lipid deposition in atherosclerosis is discussed (p. 44).

PORTER and COUCH gave whole body X-irradiation to groups of dogs in single, more than lethal, doses ranging from 450 to 700 r. Later, bone marrow from bitch puppies, given intravenously, became established in a few of the dogs, but most died of salt and fluid loss and infection. In survivors, the animals own marrow recovered to replace that transplanted (p. 52).

KIRSCHNER and GRAHAM have developed solid media for the growth, purification and maintenance of Leptospira. The media have given satisfactory results in the maintenance of sero-types of all known groups of Leptospira (p. 57).

LUNDBECK, LÖFGREN and NORDENSTAM made plasma clot cultures of explants taken from lymph glands and skin lesions in patients with sarcoidosis. They saw patchy changes in the fibroblast outgrowth from these explants, not found in controls. In these patches the cells contained eosinophilic cytoplasmic inclusions. Extracts of sarcoid lymph glands failed to produce similar lesions in cultured cells of embryo human lung (p. 61).

TREMBLAY and PEARSE using cytochemical methods have demonstrated the presence of dehydrogenase and diaphorase enzymes in parathyroid tissue of the rat. The high enzyme activity of the oxyphil cells of this tissue suggests an active secretory role rather than a stage of a degenerative process (p. 66).

FILDES and KAY have demonstrated that a number of reactions between a phage and other substances depend upon the presence of tryptophan. The part played by tryptophan in these reactions is discussed. (p. 71).

HESS, MACINTYRE, ALCOCK and PEARSE have described histochemical changes in the kidneys of rats maintained on a magnesium deficient diet. Simultaneous with a fall in plasma Mg, changes occurred in the proximal tubules made manifest by mitochondrial swelling and subsequent decline in enzyme activity. In the terminal stage intracellular calcification and necrosis was observed (p. 80).

GILLIAN CHURCHER, SHEFFIELD and WILSON SMITH describe a 2-stage method of cultivating poliomyelitis virus in ERK cells. The method yields very high infectivity titre virus fluids that can be used as flocculating antigens without prior concentration. Virulent and attenuated strains of the 3 virus types have produced effective flocculation antigens (p. 87)